Infection Biology Handbook Series
Bacterial Virulence
Basic Principles, Models and Global Approaches
Edited by Philippe Sansonetti
WILEY-BLACKWELL

VISIT…

Edited by
Philippe Sansonetti

Bacterial Virulence

Related Titles

Kaufmann, S. H. E., Walker, B. D. (eds.)

HIV and Tuberculosis

A Deadly Liaison

2010
ISBN: 978-3-527-32270-1

Schaible, U. E., Haas, A. (eds.)

Intracellular Niches of Microbes

A Pathogens Guide Through the Host Cell

2009
ISBN: 978-3-527-32207-7

Selzer, P. M. (ed.)

Antiparasitic and Antibacterial Drug Discovery

From Molecular Targets to Drug Candidates

2009
ISBN: 978-3-527-32327-2

Jungblut, P. R., Hecker, M. (eds.)

Proteomics of Microbial Pathogens

2007
ISBN: 978-3-527-31759-2

Humphery-Smith, I., Hecker, M. (eds.)

Microbial Proteomics

Functional Biology of Whole Organisms

2006
ISBN: 978-0-471-69975-0

zur Hausen, H.

Infections Causing Human Cancer

2006
ISBN: 978-3-527-31056-2

Frosch, M., Maiden, M. C. J. (eds.)

Handbook of Meningococcal Disease

Infection Biology, Vaccination, Clinical Management

2006
ISBN: 978-3-527-31260-3

Hacker, J., Dobrindt, U. (eds.)

Pathogenomics

Genome Analysis of Pathogenic Microbes

2006
ISBN: 978-3-527-31265-8

Edited by
Philippe Sansonetti

Bacterial Virulence

Basic Principles, Models and Global Approaches

WILEY-VCH Verlag GmbH & Co. KGaA

The Editor

Professor Philippe Sansonetti
INSERM U786
Institut Pasteur
28, rue du Docteur Roux
75724 Paris, Cedex 15
France

Cover
Neutrophil Granulocytes (blue) are the first line of defense against invading microorganisms. They fight bacteria (*Shigella flexneri*, red) by phagocytosis or by formation of the recently discovered Neutrophil Extracellular Traps (NETs, green) that capture and kill pathogens like bacteria, fungi and parasites. With kind permission from Volker Brinkmann, Max Planck Institute for Infection Biology, Berlin.

All books published by Wiley-VCH are carefully produced. Nevertheless, authors, editors, and publisher do not warrant the information contained in these books, including this book, to be free of errors. Readers are advised to keep in mind that statements, data, illustrations, procedural details or other items may inadvertently be inaccurate.

Library of Congress Card No.: applied for

British Library Cataloguing-in-Publication Data
A catalogue record for this book is available from the British Library.

Bibliographic information published by the Deutsche Nationalbibliothek
The Deutsche Nationalbibliothek lists this publication in the Deutsche Nationalbibliografie; detailed bibliographic data are available on the Internet at http://dnb.d-nb.de.

© 2010 WILEY-VCH Verlag GmbH & Co. KGaA, Weinheim

All rights reserved (including those of translation into other languages). No part of this book may be reproduced in any form – by photoprinting, microfilm, or any other means – nor transmitted or translated into a machine language without written permission from the publishers. Registered names, trademarks, etc. used in this book, even when not specifically marked as such, are not to be considered unprotected by law.

Cover Design Formgeber, Eppelheim
Typesetting Toppan Best-set Premedia Limited

Printing and Binding Strauss GmbH, Mörlenbach

Printed in the Federal Republic of Germany
Printed on acid-free paper

ISBN: 978-3-527-32326-5

Contents

Preface *XIII*
List of Contributors *XV*

Part I Basic Principles *1*

1 How Bacterial Pathogens were Constructed *3*
Ulrich Dobrindt and Jörg Hacker
1.1 Introduction *3*
1.2 Composition of the Flexible Gene Pool *4*
1.3 Mechanisms Involved in Genome Dynamics *6*
1.4 Bacterial Genome Optimization Using *Escherichia coli* as a Model *7*
1.5 Genome Plasticity during Infection *9*
1.6 Conclusions *10*
Acknowledgments *10*
References *11*

2 Antimicrobial Mechanisms of Neutrophils *17*
Catherine Chaput and Arturo Zychlinsky
2.1 Introduction *17*
2.2 Recruitment to Infection Sites *18*
2.3 Phagocytosis *21*
2.4 Exocytosis of Secretory Vesicles and Degranulation *22*
2.4.1 Granules and Secretory Vesicles *23*
2.4.2 Mobilization and Fusion *24*
2.5 NETs *25*
2.6 Neutrophil Function Deficiencies *26*
2.6.1 Disorders of Adhesion and Chemotaxis *26*
2.6.2 Disorders of Ingestion and Degranulation *27*
2.6.3 Disorders of Oxidative Metabolism *27*
2.7 Conclusion *28*
References *28*

Bacterial Virulence: Basic Principles, Models and Global Approaches. Edited by Philippe Sansonetti
© 2010 WILEY-VCH Verlag GmbH & Co. KGaA, Weinheim
ISBN: 978-3-527-32326-5

3 ***H. pylori* Infection – The Route from Inflammation to Cancer** *31*
Tsutomu Chiba, Hiroyuki Marusawa, Hiroshi Seno, and Norihiko Watanabe
3.1 Introduction *31*
3.2 Pathways leading to *H. pylori*-induced Gastric Carcinogenesis *31*
3.2.1 Indirect Action of *H. pylori* on Gastric Epithelial Cells through Induction of Gastritis *31*
3.2.2 Direct Action of *H. pylori* on Gastric Epithelial Cells *34*
3.2.2.1 Roles of CagA in Gastric Carcinogenesis *34*
3.2.2.2 Induction of Gene Mutations by *H. pylori* *35*
3.2.2.3 Induction of Aberrant DNA Methylation by *H. pylori* *37*
3.2.3 Relationship between Direct and Indirect Actions of *H. pylori* on Epithelial Cells in the Development of Gastric Cancer *37*
References *38*

Part II Models *43*

4 **Host–Pathogen Relationship in Skin and Soft Tissue Infections caused by Group A *streptococcus* and *Staphylococcus aureus*** *45*
Inbal Mishalian, Miriam Ravins, Moshe Baruch, Merav Persky, Ilia Belotserkovsky, and Emanuel Hanski
4.1 Introduction *45*
4.2 Restriction of GAS and SA Cutaneous Infections by Cationic Antimicrobial Peptides and Bacterial Resistance Mechanisms to these Compounds *46*
4.3 Leukocytes and Cutaneous SA and GAS Infection *47*
4.3.1 Polymorphonuclear Neutrophils (PMNs) *47*
4.3.1.1 The Interactions of SA and GAS with Innate Immunity leading to PMN Response *48*
4.3.1.2 Avoidance of SA and GAS Clearance by PMNs *50*
4.3.2 Macrophages (Mϕ) *58*
4.4 Conclusions *59*
Acknowledgments *60*
References *60*

5 **Mechanisms of Meningeal Invasion by Septicemic Extracellular Pathogens: The Examples of *Neisseria meningitidis*, *Streptococcus agalactiae* and *Escherichia coli*** *69*
Olivier Join-Lambert, Etienne Carbonnelle, Fabrice Chrétien, Sandrine Bourdoulous, Stéphane Bonacorsi, Claire Poyart, and Xavier Nassif
5.1 Introduction *69*
5.2 Bacterial Translocation through the Central Nervous System Vasculature *70*
5.2.1 Structural and Functional Heterogeneity of the Blood–CNS Interfaces *70*

5.2.1.1 The Blood–Brain Barrier Sensu Stricto *71*
5.2.1.2 The Blood-CSF Barriers *74*
5.2.2 The Passage of Extracellular Bacterial Pathogens across the Blood–CSF Barrier *75*
5.2.2.1 Where is the Blood–CSF Barrier Crossed? *75*
5.2.2.2 How do Extracellular Bacteria Breach the Blood–CSF Barrier? *76*
5.3 Neonatal Meningitis *79*
5.3.1 *Escherichia coli* Meningitis *79*
5.3.1.1 *E. coli* Strains Causing Neonatal Meningitis (ECNM) Are Oligoclonal *79*
5.3.1.2 The Specific Virulence Determinants of ECNM Strains *80*
5.3.2 Meningitis Due to Group B *Streptococcus* (GBS) *82*
5.3.2.1 GBS Strains Responsible for Neonatal Meningitis Are Oligoclonal *82*
5.3.2.2 The Specific Virulence Determinants of GBS Strains Responsible for Meningitis *83*
5.4 Cerebrospinal Meningitis *84*
5.4.1 Meningococcal Attributes that Allow Blood-borne Bacteria to Interact with Endothelial Cells *86*
5.4.2 Signaling Triggered by *N. meningitidis* that Leads to the Extravasation of Bacteria through the Brain Vessels *88*
References *91*

6 Two Important Bacterial Pathogens causing Community Acquired Pneumonia: *Streptococcus pneumoniae* and *Legionella pneumophila* *103*
Birgitta Henriques-Normark and Carmen Buchrieser
6.1 General Background *103*
6.2 *Streptococcus pneumoniae*, a Devastating Pathogen, but also a Common Colonizer of the Upper Respiratory Tract *106*
6.2.1 Epidemiology of Pneumococcal Disease and Carriage *106*
6.2.2 *In vivo* Studies of Pneumococcal Infections *108*
6.2.3 Pneumococci are Naturally Competent Bacteria *109*
6.2.4 Pneumococcal Colonization of the Respiratory Tract *110*
6.2.5 Synergism between Influenza Virus and Pneumococci *111*
6.2.6 Some Important Bacterial Virulence Factors and their Interactions with the Host *112*
6.2.7 Host Defenses against Invading Microbes *117*
6.2.8 Pneumococcal Genomes *119*
6.3 *Legionella pneumophila*, an Environmental Bacterium but also a Cause of Severe Sporadic and Epidemic Pneumonia *119*
6.3.1 Epidemiology of Legionnaires' Disease and Environmental Reservoir *119*
6.3.2 The Dot/Icm Type-IV Secretion System Central to Pathogenesis of *Legionella* *121*

6.3.3 Molecular Mimicry: the Main Virulence Strategy of *L. pneumophila* *124*
6.3.4 Implication of Eukaryotic-like Proteins in Virulence and Host Cell Modulation *125*
6.3.4.1 Entry into and Blockade of Phagosomal–Lysosomal Fusion *125*
6.3.4.2 Establishment of an ER-derived Replication Vacuole *126*
6.3.4.3 Replication in the LCV and Egress from the Host *127*
6.3.5 Evolution of the Eukaryotic-like Proteins *127*
6.3.6 *Legionella* Genomes *128*
6.3.7 Host response to *Legionella* infection *129*
6.3.7.1 The MyD88 Response is Important for the Control of *L. pneumophila* Infection *129*
6.3.7.2 Naip-5-Dependent Immune Response to Cytosolic Flagellin *130*
6.3.7.3 Dictyostelium Transcriptional Host Cell Response upon Infection *130*
6.4 Conclusions *131*
Acknowledgment *131*
References *132*

7 The *Salmonella*–Mouse Interaction: A Versatile Model to Study Bacterial Infection *139*
Jessica A. Thompson, Sophie Helaine, and David W. Holden
7.1 Introduction *139*
7.2 Identification of Virulence Genes *141*
7.2.1 *In vitro* Cell Models *141*
7.2.2 *In vivo* Screens for Virulence Genes *142*
7.3 Analyzing Gene Function *in vivo* *146*
7.3.1 Measuring the Contribution of Bacterial Genes to Virulence *146*
7.3.2 Investigating Interactions between Bacterial Virulence Genes *147*
7.3.3 Investigating Interactions between Host and Pathogen Factors *148*
7.4 In-depth Analysis of Cell Interactions within Host Tissues *153*
7.4.1 Distribution of Salmonella *153*
7.4.2 Population Dynamics of Bacteria in the Murine Host *156*
7.5 Perspectives *157*
References *158*

8 *Chlamydia*: from Molecular Insight to Therapeutic Discovery *165*
Lesley A. Ogilvie, Dagmar Heuer, and Thomas F. Meyer
8.1 Introduction *165*
8.2 *Chlamydia*: a Model Intracellular Pathogen–Host Relationship *167*
8.2.1 The Cycle of Development – *C. trachomatis* *168*
8.2.2 Genetic Determinants of Infection and Disease *172*
8.2.2.1 Chlamydial Factors *172*
8.2.2.2 Host Factors *173*
8.3 A Global Approach to Investigating the Role of Host Cell Factors during Infection *174*

8.3.1 RNAi: a New Paradigm to Study Pathogenesis *174*
8.3.2 RNAi and the *Chlamydia*–Host Interaction *178*
8.4 What can we Expect from the Global RNAi Approach? *179*
8.4.1 Basic Insights into Cellular Function and Host Susceptibility to Infection *179*
8.4.2 A New Therapeutic Concept *179*
8.5 Outlook *181*
Acknowledgments *181*
References *182*

Part III Global Approaches of Bacterial Virulence *193*

9 The Gut Microbiota and its Contribution to Homeostasis *195*
Pamela Schnupf and Philippe J. Sansonetti
9.1 Introduction *195*
9.2 Characteristics of the Gastrointestinal Microbiota *196*
9.2.1 Methods to Assess Microbial Diversity *196*
9.2.2 Diversity of the Gastrointestinal Microbiota *199*
9.2.3 Acquisition of the Gastrointestinal Microbiota *201*
9.2.4 Competition within the Intestinal Bacterial Community *202*
9.3 The Symbiosis of the Gut Microbiota and the Host *203*
9.3.1 Metabolic Functions of the Microbiota *204*
9.3.2 Development of the Gut Epithelium Architecture *205*
9.3.3 Maturation of Lymphoid Tissue *206*
9.3.4 Gut Microbiota Protects against Infection and Transmission of Pathogens *207*
9.3.5 Gut Microbiota induces Immune Tolerance against Itself, the Host, and Environmental Antigens *207*
9.3.6 Enteropathogens Exploit Inflammation for their Own Benefit *209*
9.4 Conclusion *210*
Abbreviations *211*
References *211*

10 Anatomy of the Gut Barrier and Establishment of Intestinal Homeostasis *215*
Gernot Sellge, Pamela Schnupf, and Philippe J. Sansonetti
10.1 Introduction *215*
10.2 Anatomical, Physiological and Immunological Properties of the Gut Barrier *216*
10.2.1 The Intestinal Lumen and the Oxygen Availability at the Tips of the Villi *218*
10.2.2 Goblet Cells and the Mucus Layer *218*
10.2.3 The Mucosal Epithelium: Physical Barrier and Immune Function *220*
10.2.4 Intestinal Crypts, Paneth Cells and Antimicrobials *222*
10.2.5 Innate Immune Cells of the Lamina Propria *224*

10.2.6 Antigen Sampling in the Gut *226*
10.2.7 The Protective IgA Response *228*
10.2.8 The Balance between Regulatory and Effector T Cells in the Gut *230*
10.3 Sensing, Signaling and Responding to the Microbiota to Establish a Symbiotic Relationship and Gut Homeostasis *232*
10.3.1 Controlling Pro-inflammatory Antigens *233*
10.3.2 Sensing the Presence of Microbes from Afar *235*
10.3.3 Sensing the Presence of Microbes at the Cell Surface *236*
10.3.4 Sensing the Presence of Microbes Intracellularly *237*
10.3.5 The Master Regulator of Intestinal Inflammation and Homeostasis: The NF-κB Signaling Pathway *238*
10.4 Conclusion *241*
Abbreviations *243*
References *243*

11 Dynamic Imaging Technologies to Explore Infectious Processes at the Cellular, Tissue and Organ Level *251*
Jost Enninga, Regis Tournebize, Keira Melican, and Agneta Richter-Dahlfors
11.1 Introduction to Imaging of Host–Pathogen Interactions *251*
11.2 Imaging of Infections *253*
11.2.1 Airway and Lung Infections *254*
11.2.2 Infections of the Gastrointestinal Tract *256*
11.2.3 Urinary Tract Infections *262*
11.2.4 Meningitis *267*
11.3 Clinical Implications *268*
11.4 Future Technological Developments *270*
References *271*

12 The Issue of Species Specificity of Bacterial Infection, How to Address It Experimentally *279*
Olivier Disson, Pascale Cossart, and Marc Lecuit
12.1 Introduction *279*
12.2 Molecular Aspects of Species Specificity *280*
12.3 Modelization of Bacterial Invasion Using Human Tissues *Ex Vivo* *281*
12.3.1 *Ex Vivo* Infection of Blood and Body Fluids *281*
12.3.2 *Ex Vivo* Infection of Solid Tissues *281*
12.4 *In Vivo* Modeling and the Quest for Relevant Animal Models *282*
12.4.1 Non-human Primates *282*
12.4.2 Non-primate Mammals *282*
12.5 *In Vivo* Modeling with a Focus on the Pathogen rather than the Host *283*
12.5.1 Studying a Closely-related Pathogen *283*
12.5.2 Modifying the Bacterium *284*

12.6 *In Vivo* Modeling: use of Human Xenografts to generate 'Humanized' Animal Models *284*
12.7 Genetic Engineering to Circumvent Species-specificity and Generate 'Humanized' Animal Models for Human Infectious Diseases *286*
12.7.1 Transgenesis *286*
12.7.2 Knock-in *287*
12.8 *Listeria* as a Model of Intestinal and Materno-Fetal Infections *287*
12.8.1 InlA and InlB are Involved in *Lm* Invasion of Non-phagocytic Cells *In Vitro* in a Species-Specific Manner *288*
12.8.2 Gastroenteritis *290*
12.8.3 Materno-Fetal Infections *292*
12.9 *Streptococcus pyogenes* Infections: from Colonization of Host Epithelial Barriers to Systemic Infection *296*
12.9.1 *Streptococcus pyogenes* (GAS) as a Model of Pharyngitis and Skin Infections *296*
12.9.2 Skin Infections *296*
12.9.3 Models of Pharyngitis *299*
12.9.3.1 *Ex Vivo* *299*
12.9.3.2 *In Vivo* *299*
12.9.4 CbpA, the Adhesin of *Streptococcus pneumoniae* Involved in Species Specificity for Humans *300*
12.10 *Neisseria meningitidis* and Cerebrospinal Meningitis *301*
12.10.1 *Neisseria meningitidis* *301*
12.10.2 CD46 and Meningitis *301*
12.10.3 Other Molecular Interactions at the Blood–Brain Barrier Level *303*
12.10.4 Other Host-specific Interactions which occur during Meningococcal Infection *303*
12.11 Concluding Remarks *303*
References *305*

Index *311*

Preface

Microbial virulence is a fast-moving field. From molecular genetics to cell biology and signaling, from genomics to dynamic imaging of infectious processes both *in vitro* and *in vivo*, from immunology to inflammation and cancer, few concepts and techniques in life sciences have not been applied, over the last years, to better decipher the detailed mechanisms of infections. A single volume could not account for these amazing progresses that encompass both sub-cellular dissection of the signals elicited by the engagement of cell targets by microbial effectors, and supra-cellular integration of these microbe-cell cross-talks in a global scheme of tissue or organ infection. This transition from cellular microbiology to tissue microbiology is possibly one of the most striking recent trends in our discipline. It has been made possible by the combination of improved animal models of infection, including transgenic and KO mice and real time imaging methods such as two-photon microscopy. Tissue microbiology, as it currently evolves, is also creating a very fertile ground for collaborations between microbiologist and immunologists. Interactions started with the deciphering of innate mechanisms of host defenses in the presence of pathogens, particularly how the host can discriminate among commensal microorganisms and true pathogens in order to initiate an inflammatory response that is adapted to the level of the threat. Interactions continue with the molecular and cellular analysis of interference mechanisms between pathogens and adaptive immune responses. A scheme emerges according to which, at key anatomic barriers of the host body, sensing mechanisms discriminate pathogens from commensals, elicit an inflammatory response, both humoral and cellular, that is aimed at eradicating the aggressive pathogen, possibly at the cost of significant tissue damage and fatal rupture of this barrier. The pathogen itself modulates this response, thereby achieving better colonization and invasion of this barrier, thus of the host. This Yin and Yang strategy of pathogenic microbes reflects a long co-evolution between the eukaryotic and prokaryotic kingdoms. It can be read in the microbial genomes through recognition of pathogenicity genes and islands reflecting the "construction" of these pathogens under the host selective pressure. Evolution of pathogens from their commensal ancestors is another illustration of Darwin's theory.

This volume gathers among the best experts in the field of bacterial pathogenesis to illustrate the above statements. Following introductory chapters on the genetic

Bacterial Virulence: Basic Principles, Models and Global Approaches. Edited by Philippe Sansonetti
© 2010 WILEY-VCH Verlag GmbH & Co. KGaA, Weinheim
ISBN: 978-3-527-32326-5

identity of pathogens and new developments in the understanding of the antimicrobial strategies of a first-line defense cell, the neutrophil, the book goes on describing paradigms of rupture, colonization - invasion and inflammatory injury of major barriers of the body. These paradigms illustrate the integration of cellular microbiology, tissue microbiology, and immunopathology in acute and chronic modes of infection, the latter possibly leading to cancer as is the case of gastric infection by *Helicobacter pylori*. Hopefully, this angle provides a very dynamic view of infectious processes as they develop. The last part should be seen as an opening on two novel developments that will be increasingly be part of the study of infections, i.e. the understanding of the mechanisms of tolerance of our commensal microbiota and how the rupture of tolerogenic signals may participate to diseases, and the molecular bases of species specificity of pathogens, the subversion of which is central to the concept of emergence of new infectious diseases.

Paris, December 2009 *Philippe Sansonetti*

List of Contributors

Moshe Baruch
The Hebrew University-Hadassah
Medical School
Institute of Microbiology
Jerusalem 91120
Israel

Ilia Belotserkovsky
The Hebrew University-Hadassah
Medical School
Institute of Microbiology
Jerusalem 91120
Israel

Stéphane Bonacorsi
Université Paris Diderot
Equipe d'accueil
EA3105, Paris
France

Sandrine Bourdoulous
Université Paris Descartes
Institut Cochin
Département de Biologie Cellulaire
INSERM
Unité 567
CNRS
UMR8104, Paris
France

Carmen Buchrieser
Institut Pasteur
UP Biologie des Bactéries
Intracellulaires
Department Genomes and Genetics
25, rue du Dr. Roux
75724 Paris Cedex 15
France

Etienne Carbonnelle
Université Paris Descartes
INSERM U570
156 rue de Vaugirard
75015 Paris
France

Catherine Chaput
Max-Planck Institute for Infection
Biology
Department of Cellular Microbiology
Charitéplatz 1
Berlin, 10 117
Germany

Tsutomu Chiba
Kyoto University
Department of Gastroenterology and
Hepatology
Graduate School of Medicine
Kwahara-cho 54
Shogoin, Sakyo
Kyoto 606-8507
Japan

Bacterial Virulence: Basic Principles, Models and Global Approaches. Edited by Philippe Sansonetti
© 2010 WILEY-VCH Verlag GmbH & Co. KGaA, Weinheim
ISBN: 978-3-527-32326-5

Fabrice Chrétien
Université Paris 12 Val-de-Marne
INSERM
Unité 955
Département de Pathologie
hopital Henri Mondor
APHP, Créteil
94000, Paris
France

Pascale Cossart
Institut Pasteur
Unité des Interactions
Bactéries-Cellules
INSERM U604
25 Rue du Docteur Roux
75724 Paris cedex 15
France

Olivier Disson
Institut Pasteur
Groupe "Microorganismes et Barrières de l'hôte"
INSERM Avenir U604
25 Rue du Docteur Roux
75724 Paris cedex 15
France

Ulrich Dobrindt
Universität Würzburg
Institut für Molekulare Infektionsbiologie
Röntgenring 11
97070 Würzburg
Germany

Jost Enninga
Institut Pasteur
Groupe 'Dynamique des interactions hôte-pathogène'
25 rue du Dr. Roux
75724 Paris Cedex 15
France

Jörg Hacker
Robert Koch-Institut
Am Nordufer 20
13353 Berlin
Germany

Emanuel Hanski
The Hebrew University-Hadassah Medical School
Institute of Microbiology
Jerusalem 91120
Israel

Sophie Helaine
Imperial College London
Department of Microbiology
CMMI
Flowers Building
Armstrong Road
London SW7 2AZ
UK

Birgitta Henriques-Normark
Swedish Institute for Infectious Disease Control
Department of Bacteriology
SE 171 82, Solna
Sweden
Department of Microbiology, Tumur Biology and Cell Biology
Karolinska Institutet
SE 171 77, Stockholm
Sweden

Dagmar Heuer
Max Planck Institute for Infection Biology
Department of Molecular Biology
Charitéplatz 1
10117 Berlin
Germany

David W. Holden
Imperial College London
Department of Microbiology
CMMI
Flowers Building
Armstrong Road
London SW7 2AZ
UK

Olivier Join-Lambert
Université Paris Descartes
INSERM U570
156 rue de Vaugirard
75015 Paris
France

Marc Lecuit
Institut Pasteur
Groupe "Microorganismes et Barrières de l'hôte"
INSERM Avenir U604
CNR Listeria
25 Rue du Docteur Roux
75724 Paris cedex 15

Université Paris-Descartes
Hôpital Necker-Enfants malades
75015 Paris
France

Hiroyuki Marusawa
Kyoto University
Department of Gastroenterology and Hepatology
Graduate School of Medicine
Kwahara-cho 54
Shogoin, Sakyo
Kyoto 606-8507
Japan

Keira Melican
Karolinska Institutet
Department of Neuroscience
Retzius väg 8
17177 Stockholm
Sweden

Thomas F. Meyer
Max Planck Institute for Infection Biology
Department of Molecular Biology
Charitéplatz 1
10117 Berlin
Germany

Inbal Mishalian
The Hebrew University-Hadassah Medical School
Institute of Microbiology
Jerusalem 91120
Israel

Xavier Nassif
Université Paris Descartes
INSERM U570
156 rue de Vaugirard
75015 Paris
France

Lesley A. Ogilvie
Max Planck Institute for Infection Biology
Department of Molecular Biology
Charitéplatz 1
10117 Berlin
Germany

Merav Persky
The Hebrew University-Hadassah Medical School
Institute of Microbiology
Jerusalem 91120
Israel

Claire Poyart
Université Paris Descartes
Institut Cochin
Département de Maladies Infectieuses
INSERM
Unité 567
CNRS
UMR8104, Paris
France

Miriam Ravins
The Hebrew University-Hadassah
Medical School
Institute of Microbiology
Jerusalem 91120
Israel

Agneta Richter-Dahlfors
Karolinska Institutet
Department of Neuroscience
Retzius väg 8
17177 Stockholm
Sweden

Philippe J. Sansonetti
Institut Pasteur
Unite de Pathogénie Microbienne
Moléculaire
INSERM U786
28 Rue du Docteur Roux
75724 Paris cedex 15
France

Pamela Schnupf
Institut Pasteur
Unite de Pathogénie Microbienne
Moléculaire
INSERM U786
28 Rue du Docteur Roux
75724 Paris cedex 15
France

Gernot Sellge
Institut Pasteur
Unite de Pathogénie Microbienne
Moléculaire
INSERM U786
28 Rue du Docteur Roux
75724 Paris cedex 15
France

Hiroshi Seno
Kyoto University
Department of Gastroenterology and
Hepatology
Graduate School of Medicine
Kwahara-cho 54
Shogoin, Sakyo
Kyoto 606-8507
Japan

Jessica A. Thompson
Imperial College London
Department of Microbiology
CMMI
Flowers Building
Armstrong Road
London SW7 2AZ
UK

Regis Tournebize
Institut Pasteur
Unité 'Pathogénie microbienne
moléculaire'
28 rue du Dr. Roux
75724 Paris Cedex 15
France
Institut Pasteur
Unité INSERM U786
28 rue du Dr Roux
75724 Paris Cedex 15
France

Norihiko Watanabe
Kyoto University
Department of Gastroenterology and Hepatology
Graduate School of Medicine
Kwahara-cho 54
Shogoin, Sakyo
Kyoto 606-8507
Japan

Arturo Zychlinsky
Max-Planck Institute for Infection Biology
Department of Cellular Microbiology
Campus Charité Mitte
Schumannstraße 21/22
Berlin, 10117
Germany

Part I
Basic Principles

Bacterial Virulence: Basic Principles, Models and Global Approaches. Edited by Philippe Sansonetti
© 2010 WILEY-VCH Verlag GmbH & Co. KGaA, Weinheim
ISBN: 978-3-527-32326-5

1
How Bacterial Pathogens were Constructed

Ulrich Dobrindt and Jörg Hacker

1.1
Introduction

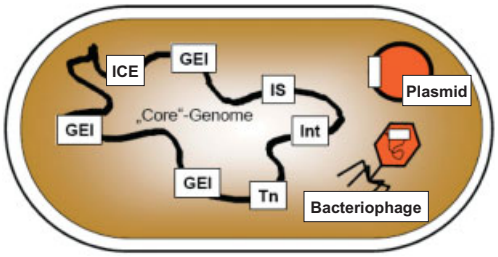
The complete genome sequences of multiple variants of almost all bacterial pathogens have already been published. There is large variation in size and content of bacterial genomes between different genera and species but also among strains of the same species. The adaptive capability ('versatility') of bacteria directly correlates with genome size [1]. Genome optimization in bacteria with an intracellular parasitic lifestyle, for example, *Chlamydia trachomatis* and *Rickettsia prowazeckii* (genome size 1.04 Mb and 1.11 Mb, respectively) which live in a constant and rich environment, involves marked genome reduction due to the loss of genetic information coding for traits and functions that could be obtained from the host.

Consequently, the genomes of such bacteria living in close association with their host are much smaller than those of environmental bacteria that may be facultative pathogens, e.g. *Pseudomonas aeruginosa* (genome size 6.3 Mb), as these organisms have to live under different and variable growth conditions.

The analysis of these sequences revealed that genomes of pathogenic microbes, but also of microbes from other sources, may comprise different numbers of circular or linear chromosomes, extrachromosomal linear or circular replicons as well as different combinations thereof. The genome of *Vibrio cholerae*, the causative agent of cholera, contains two chromosomes, both of them encode important functions [2, 3]. Most prokaryotes, however, contain a single circular chromosome which comprises genes providing the backbone of genetic information required for essential cellular processes and which is not transferable *per se*. The arrangement of genes on the chromosome is characterized by a frequently clustered organization or a close link between functionally related genes. Genes located on the 'core' part of the chromosome exhibit a relatively homogenous G+C content and a specific codon usage. The genomic organization of closely related bacteria is very similar [4].

The structure of the 'core' regions of the genome, however, is often interrupted by the presence of DNA stretches harboring genes with a G+C content and a codon

Bacterial Virulence: Basic Principles, Models and Global Approaches. Edited by Philippe Sansonetti
© 2010 WILEY-VCH Verlag GmbH & Co. KGaA, Weinheim
ISBN: 978-3-527-32326-5



Plasmids
Bacteriophages
Genomic islands (GEI)
Integrative and conjugative elements (ICE)
Integrons (Int)
(Conjugative)Transposons (Tn), IS elements (IS)

Figure 1.1 Composition of a bacterial genome. The core genome is shown together with the various elements of the flexible gene pool.

usage which differs from those of the 'core' genome. This 'flexible' gene pool consists of strain-specific 'assortments' of genetic information mainly represented by mobile genetic elements, such as plasmids, bacteriophages, genomic/pathogenicity islands (GEIs/PAIs), integrons, IS-elements and transposons (see Figure 1.1 and Table 1.1), which provide additional traits contributing to the adaptation of microbes under certain environmental conditions, e.g. resistance to antibiotics, production of toxic compounds as well as other virulence factors [5].

1.2 Composition of the Flexible Gene Pool

IS elements and transposons are considered to be 'jumping genes' as they can frequently change their chromosomal localization. Generally, transposable elements fall into three groups: (i) insertion sequences (relatively small mobile genetic entities that generally encode no functions other than those involved in their mobility and exhibit short terminal inverted repeat sequences), (ii) composite transposons (flanked by insertion sequences at both ends), and (iii) non-composite transposons (lack flanking insertion sequences). Many transposons carry antibiotic resistance genes [6].

Integrons can also jump and may carry determinants for antibiotic resistances and other properties. They can be considered as a natural genetic engineering system because they are assembly platforms, i.e. DNA elements that acquire genes embedded in exogenous gene cassettes and convert them to functional genes by ensuring their correct expression. They were initially identified because of their important role in the spread of antibiotic-resistance determinants. Meanwhile,

Table 1.1 Mobilizable and transferable accessory genetic elements of the flexible gene pool that contribute to genome plasticity.

DNA element	Size	Characteristics
Pathogenicity/ Genomic Island	20–200 kb	Modular, mosaic-like composition, site-specific insertion into tRNA genes
Plasmids	2–200 kb	Modular, mosaic-like composition, sometimes chromosomal insertion possible, can transfer transposons and IS elements
Integrative and conjugative elements	30–150 kb	Site-specific insertion into tRNA genes, modular composition
Prophages	40–100 kb	Site-specific insertion frequently into tRNA genes
(Conjugate) Transposons	20–100 kb	Mainly in Gram-positive bacteria
(Super-)Integrons	~100 kb	Mainly in Gammaproteobacteria, many promoter-less gene cassettes
Transposons	2.5–25 kb	Flanked by IS elements
Insertion sequences	0.7–2.5 kb	Encode only their integrase

larger integron structures, termed superintegrons, have been discovered which further support their importance in bacterial genome evolution. The latter contain hundreds of accessory genes and constitute a significant fraction of the genomes of many bacterial species including important intestinal pathogens such as *Vibrio cholerae* [7].

Transferable genetic elements such as bacteriophages and plasmids can function as vehicles laterally transporting genetic information, thus playing an important role in bacterial evolution. Bacteriophages may carry genes that bring about new functions or modify existing ones upon becoming part of the host genome. Bacteriophages often carry toxin genes, e.g. the cholera toxin determinant, the genes encoding diphtheria toxin or the Shiga toxins. Plasmids are circular, self-replicating DNA molecules. They are autonomous molecules and exist in cells as extrachromosomal genomes, although some plasmids can be inserted into a bacterial chromosome. The role of transferable elements as vectors as well as the constantly ongoing recombination between different mobilizable and transferable DNA elements is exemplified for example by the description of the association of multi-drug resistance and virulence determinants in form of a large virulence plasmid in multi-drug-resistant *Salmonella enterica* serotype Typhimurium that carries several virulence genes and two class 1 integrons [8] or a multi-resistance plasmid of *Klebsiella pneumoniae* that resembles enterobacterial integrative and conjugative elements as well as plasmids found in *Yersinia pestis* [9, 10].

Genomic islands (GEIs) are large chromosomal sectors which are part of the flexible gene pool as they represent formerly transferred DNA regions. Pathogenicity islands (PAIs) are particular GEIs present in pathogenic bacteria but absent in the majority of closely related non-pathogenic variants. They carry one or more virulence-associated gene(s) and are frequently associated with tRNA genes as well as flanked by repeat structures. As PAIs are often unstable and as they contain mobility genes coding for integrases or transposases they contribute to the dynamic character of chromosomes in pathogens [5, 11]. Certain conjugative and self-transmissible genetic elements exhibit features of plasmids and bacteriophages as they can be transferred by conjugation and can integrate into the bacterial chromosome. Such integrative and conjugative elements (ICEs) may function as progenitors of some PAIs/GEIs during genome evolution [12].

Accessory genetic elements like transposons, integrons, insertion elements and genomic or pathogenicity islands (GEIs, PAIs) represent major constituents of the flexible gene pool. The genomic islands are seldom fixed but rather bear the potential for ongoing rearrangements, deletions and insertions. As a result, the stable chromosomal backbone and the flexible gene pool are constantly undergoing repeated insertions and deletions leading to new variants, pathotypes and over a long-term process to new species. Thus, such genomes are composed of clonally evolving DNA regions that are periodically disrupted due to exchange of already existing gene blocks by homologous recombination, and insertion of horizontally acquired DNA segments. The majority of strain- or pathotype-specific regions have accumulated over time by repeated horizontal gene transfer, frequently with successive transfers of different elements into identical loci of the core chromosome. The existence of so many different horizontally-acquired sequences in genomic islands differentiating closely related strains indicates that many of them are only temporarily present in the genome or provide a specific advantage to the individual lifestyle of particular strains.

1.3 Mechanisms Involved in Genome Dynamics

Different mechanisms contribute synergistically to dynamic bacterial genome evolution: first, point mutations can be accumulated and result in the diversification of genes. Second, variation in the bacterial genome organization results from transposition, site-specific as well as homologous recombination of DNA regions. Repeated sequences, notably mobile DNA elements, play a major role in the overall genome plasticity as they facilitate recombination and these DNA rearrangements may cause relocation or deletion of genomic regions. DNA recombination also causes gene duplication. Duplicated genes can evolve as orthologues or by divergent evolution as paralogues. Additionally, foreign genetic material is acquired by horizontal gene transfer. This acquisition of genetic material results in extremely dynamic genomes in which substantial amounts of DNA are introduced into and deleted from the chromosome. The extent of gene acquisition in bacteria differs

considerably between different genera and species and may be responsible for up to 15% of the complete genome sequence [13]. The combination of such evolutionary mechanisms during prokaryotic genome evolution can be nicely illustrated by the shuffling and disruption of operons in many bacteria due to rearrangements and gene transfer [14].

1.4 Bacterial Genome Optimization Using *Escherichia coli* as a Model

The species *Escherichia coli* comprises pathogenic and non-pathogenic variants [5, 15, 16]. Pathogenic isolates may cause infections of the intestine as well as extraintestinal infections such as urinary tract infections, sepsis and meningitis. Commensal *E. coli* have the capacity to colonize the intestine of humans and many animals without causing any harm.

The pheno- and genotypic variability of pathogenic and commensal *E. coli* correlate with their genome content. *E. coli* genomes vary in size from 4.6 to 5.6 Mb [17]. These size differences among individual *E. coli* genomes indicate the presence of different amounts of strain-specific genetic information, which may represent up to 30% of the complete genome content. Genes for many virulence traits as well as antibiotic resistance genes of IPEC (intestinal porcine epithelial cell line-1) and ExPEC, especially those characteristic of the different pathotypes, may be encoded on mobile and accessory genetic elements, e.g. genomic and pathogenicity islands or transposons [5, 18–21], plasmids and bacteriophages [22–26].

Extraintestinal pathogenic *E. coli* (ExPEC) are epidemiologically and phylogenetically distinct from many commensal strains as well as from IPEC. A variety of virulence factors directly contribute to pathotype-specific disease and their distribution is thus restricted to the corresponding pathotypes. For instance, the ETT-1 type III secretion system and its translocated effectors are usually indicative of enterohemorrhagic *E. coli* (EHEC) and enteropathogenic *E. coli* (EPEC). The heat-stable or heat-labile enterotoxins are characteristic of enterotoxigenic *E. coli* (ETEC) [16]. Certain invasion genes such as *ibeA* as well as the K1 capsule determinant are frequently present in invasive ExPEC [27]. In many cases, however, ExPEC and commensal *E. coli* share a large fraction of their genome [15, 28, 29]. There are also many so-called virulence-associated factors in ExPEC such as colicins, certain fimbriae, siderophore systems and toxins [15, 30–32] that have probably evolved to enhance survival in the gut and/or transmission between hosts, and therefore will be shared with some commensal strains and sometimes even with IPEC.

As many *E. coli* virulence-associated genes may be located on transmissible genetic elements such as bacteriophages, plasmids or transposons, individual DNA regions can be exchanged between the chromosome and mobile genetic elements with the capacity to integrate into and excise from the bacterial chromosome. Accordingly, several identical or closely related virulence determinants can be found on the chromosome or on mobile DNA elements.

So-called colicin plasmids represent an interesting example of such mobile elements which in large part exhibit considerable sequence similarity to PAIs in *E. coli* and contribute to PAI evolution and the spread of virulence traits among individual strains. Large colicin plasmids are found primarily in virulent, mainly septicemic *E. coli* strains and they seem to be a characteristic marker for avian pathogenic *E. coli* (APEC), causing systemic infections in poultry for example. The genetic structure of such plasmids from APEC highly resembles that of other large colicin and related plasmids and several PAIs of *E. coli* [33] as they carry several genes that have been previously associated with APEC virulence such as those coding for colicins, toxins and factors involved in serum resistance. It also contains several operons associated with iron acquisition including the aerobactin system (*iuc/iut*), the *iro* determinant coding for salmochelin, the *sit* operon, coding for an ABC transport system involved in iron and manganese transport and the *eitA-D* genes that code for a putative iron transport system. The operons coding for the siderophore systems *sit*, *iut* and *iro* as well as the *iss* gene involved in serum resistance can be found on the bacterial chromosome as well as on the colicin plasmids [34]. In APEC, these determinants are exclusively found on colicin plasmids whereas in other pathogenic enterobacteria they are frequently located on chromosomal PAIs [33, 35].

As already mentioned, many PAI regions exhibit notable homology to fragments of mobile genetic elements such as bacteriophages and virulence plasmids. In addition, multiple copies of accessory DNA elements in one genome facilitate homologous recombination within one or between different islands or horizontally-acquired DNA elements thus leading to rearrangements, deletions and acquisition of 'foreign' DNA. Consequently, many PAIs have a mosaic-like modular structure. Although many of them superficially resemble each other with respect to the presence and/or genetic linkage of certain virulence determinants, PAI composition, structural organization and chromosomal localization can be highly variable even among strains of the same patho- or serotype [36, 37]. A recent comparative genomic and phylogenetic analysis of 20 commensal and pathogenic *E. coli* strains indicated that genome variability within the *E. coli* species is restricted to a small number of conserved chromosomal positions. These hot spots of gene acquisition and loss correspond to regions of abundant and parallel insertions and deletions of DNA and can comprise between 10 and 157 genes [38]. Such large genomic regions seem to be more permissive to the insertion/deletion of accessory DNA elements as once they are chromosomally inserted they serve as preferred regions for other insertion/deletion events. The resulting larger regions are then distributed within *E. coli* by homologous recombination between their flanking genes rather than by dissemination of the individual accessory elements comprising these genomic hot spots [38].

It has been shown that the virulence features of pathogenic microbes may not only vary between different strains, but also between different time points of infection within a single strain. To analyze the flexibility of PAIs, we used the method of 'island probing' [39–41]. Using this method it was possible to calculate the deletion rate of respective PAIs. With a deletion rate of 10^{-3} to 10^{-4}, they can be excised

from their respective genome. The integrases, which are encoded by the respective PAIs are involved in this deletion processes. Generally they act highly specifically at their respective target sites [42, 43], but may sometimes also have the capacity to engage in 'cross-talk' and thus also to be involved in the excision of another island. PAI deletion may be beneficial in the course of an infection as later stages of infection may develop into chronic infections. Under these conditions, several virulence-associated traits, especially toxins, may be disadvantageous.

1.5 Genome Plasticity during Infection

Variation in the bacterial genome organization results in large part from transposition and homologous recombination. Repeated sequences, notably mobile DNA elements, play a major role in genome plasticity. Repeated DNA sequences play a primordial role in the overall genome plasticity as they are frequently involved in recombination events. It is postulated, and also frequently observed, that 'useless' or somehow deleterious genes are lost from bacterial genomes (genome reduction). Without a positive selection pressure, a gene will be lost rapidly. This loss will be even more rapid if the particular gene is part of a mobile DNA element [44].

Recent studies suggest that genome plasticity seems to be accelerated under *in vivo* conditions as it may facilitate adaptation to various host conditions. During infection, a strong selection pressure is exerted on the pathogen, leading to variations in the clonal lineages. Pheno- and genotypic changes have been reported for consecutive *Escherichia coli* isolates from recurrent bacteremia cases. In some patients, *E. coli* isolates from consecutive recurrent bacteremia episodes exhibited an alteres ability to express long chain LPS, capsule or flagella [45]. *E. coli*, the major cause of urinary tract infections (UTI), may also cause asymptomatic bacteriuria (ABU), i.e. a carrier state without causing symptoms. This resembles a state of commensalism with a bacterial monoculture rather than a complex flora. Accordingly, ABU is an interesting model in which to study mechanisms of commensalism and the driving forces within the pathogen and host. Geno- and phenotypic analyses of ABU isolates demonstrated that many ABU strains arose from virulent variants by gene loss. It has been suggested that attenuation may constitute a general mechanism for mucosal pathogens to evolve towards commensalism [46].

For example, phage mobilization contributes significantly to genome alteration in *Staphylococcus aureus* cystic fibrosis isolates during infection. Such a genome alteration could be linked to bacteriophage mobilization, phage conversion or deletion. [47]. Similarly, a recent complete sequence and comparative analysis of the genomes of two representative *P. aeruginosa* strains isolated from cystic fibrosis (CF) patients with that of other *P. aeruginosa* isolates indicated that niche adaptation is a major evolutionary force influencing the composition of bacterial genomes. Unlike the genome reduction seen in host-adapted bacterial pathogens, the genetic

capacity of *P. aeruginosa* is determined by the ability of individual strains to acquire or discard genomic segments, giving rise to strains with customized genomic repertoires [48].

The gastric pathogen *Helicobacter pylori* shows immense genetic variability in gene content and at the sequence level within human populations [49–51]. Comparison of the genome content of 21 closely related pairs of isolates taken from the same patient at different time points showed that the great majority of genetic changes were due to homologous recombination. These results suggest that adaptation of *H. pylori* to the host individual may principally occur through sequence changes rather than loss or gain of genes [52]. Nevertheless, comparative genomic analysis of chronic atrophic gastritis (ChAG) *H. pylori* isolates also suggests that certain genes may have been lost or gained during progression to adenocarcinoma. Furthermore, adaptation to ChAG also includes alteration in the expression of genes encoding components of metal uptake and utilization pathways, outer membrane proteins and virulence factors [53].

1.6 Conclusions

Comparative genomics indicate that a permanent process of construction and deconstruction of microbial genomes represents an important mechanism of genome evolution, both in short-term intervals (microevolution) as well as in long-term periods (macroevolution). These processes, which have been analyzed in detail for *E. coli*, are also valid for other pathogenic as well as non-pathogenic microbes. Pathogenicity or genomic islands, may exhibit a particular 'life cycle' of deconstruction and reconstruction: accessory genetic elements can integrate into the core genome preferentially in a site-specific manner (see Figure 1.2). Following 'reduction', particular islands lose gene clusters whose products do not contribute to better survival and transmission of the strain. Following re-integration of additional transposons or IS-elements, classical PAIs will be generated. Islands can delete from the chromosome, but they have the capacity to re-integrate and transfer. It can therefore be concluded that the analysis of the mechanisms involved in destruction and restructuring of bacterial genomes results in a model of the evolutionary processes of microbes in general.

Acknowledgments

We thank Claudia Borde for help in the preparation of the manuscript. Our own work is supported by the 'Deutsche Forschungsgemeinschaft' (SFB 479) and the 'Bavarian Research Foundation'. This work was carried out within the framework of the European Virtual Institute for Functional Genomics of Bacterial Pathogens (CEE LSHB-CT-2005-512061) and the ERA-NET Pathogenomics project 'Deciphering the intersection of commensal and extraintestinal pathogenic *E. coli*' (Grant no. 0313937A).

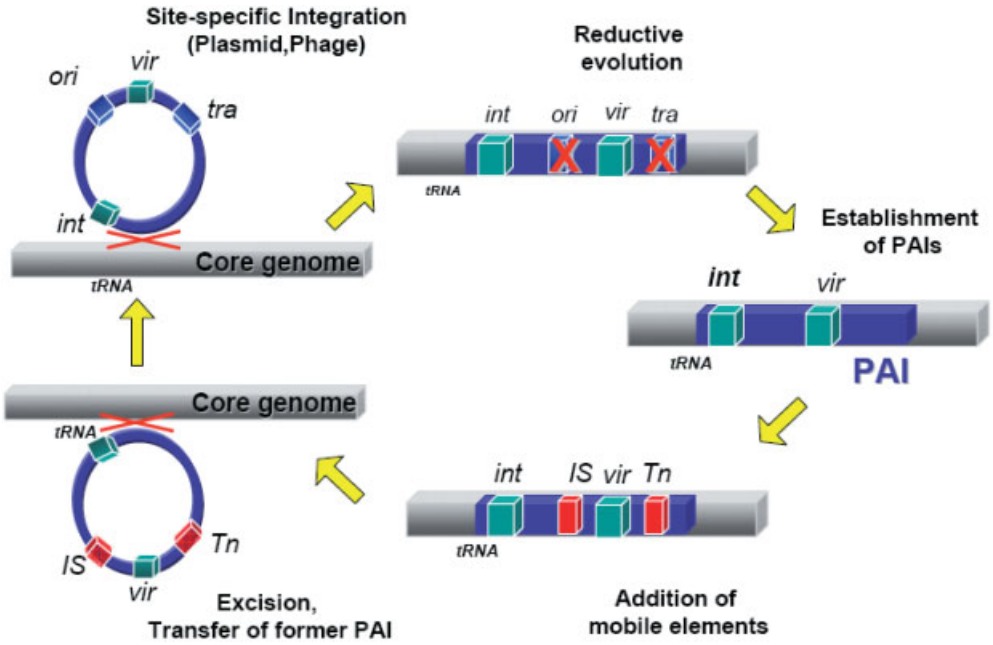


Figure 1.2 Processes of deconstruction and reconstruction of pathogenicity islands. The 'life cycle' shows the development of pathogenicity islands as well as the excision and integration processes involved. ori, origin of replication; vir, virulence gene; tra, transfer gene; int, integrase gene; IS, insertion sequence; Tn, transposon.

References

1 Mira, A., Klasson, L., and Andersson, S.G. (2002) Microbial genome evolution: sources of variability. *Curr. Opin. Microbiol.*, **5**, 506–512.

2 Casjens, S. (1998) The diverse and dynamic structure of bacterial genomes. *Annu. Rev. Genet.*, **32**, 339–377.

3 Heidelberg, J.F., Eisen, J.A., Nelson, W.C., Clayton, R.A., Gwinn, M.L., Dodson, R.J., Haft, D.H., Hickey, E.K., Peterson, J.D., Umayam, L., Gill, S.R., Nelson, K.E., Read, T.D., Tettelin, H., Richardson, D., Ermolaeva, M.D., Vamathevan, J., Bass, S., Qin, H., Dragoi, I., Sellers, P., McDonald, L., Utterback, T., Fleishmann, R.D., Nierman, W.C., White, O., Salzberg, S.L., Smith, H.O., Colwell, R.R., Mekalanos, J.J., Venter, J.C., and Fraser, C.M. (2000) DNA sequence of both chromosomes of the cholera pathogen *Vibrio cholerae*. *Nature*, **406**, 477–483.

4 Holm, L. (1986) Codon usage and gene expression. *Nucleic Acids Res.*, **14**, 3075–3087.

5 Dobrindt, U., Hochhut, B., Hentschel, U., and Hacker, J. (2004) Genomic islands in pathogenic and environmental microorganisms. *Nat. Rev. Microbiol.*, **2**, 414–424.

6 Craig, N., Craigie, R., Gellert, M., and Lambowitz, A. (2002) *Mobile DNA II*, ASM Press, Washington D.C.

7 Mazel, D. (2006) Integrons: agents of bacterial evolution. *Nat. Rev. Microbiol.*, **4**, 608–620.

8 Villa, L., and Carattoli, A. (2005) Integrons and transposons on the *Salmonella enterica* serovar typhimurium

virulence plasmid. *Antimicrob. Agents Chemother.*, **49**, 1194–1197.

9 Lin, T.L., Lee, C.Z., Hsieh, P.F., Tsai, S.F., and Wang, J.T. (2008) Characterization of integrative and conjugative element ICE*Kp1*-associated genomic heterogeneity in a *Klebsiella pneumoniae* strain isolated from a primary liver abscess. *J. Bacteriol.*, **190**, 515–526.

10 Soler Bistué, A.J., Birshan, D., Tomaras, A.P., Dandekar, M., Tran, T., Newmark, J., Bui, D., Gupta, N., Hernandez, K., Sarno, R., Zorreguieta, A., Actis, L.A., and Tolmasky, M.E. (2008) *Klebsiella pneumoniae* multiresistance plasmid pMET1: similarity with the *Yersinia pestis* plasmid pCRY and integrative conjugative elements. *PLoS ONE*, **3**, e1800.

11 Schmidt, H., and Hensel, M. (2004) Pathogenicity islands in bacterial pathogenesis. *Clin. Microbiol. Rev.*, **17**, 14–56.

12 Schubert, S., Dufke, S., Sorsa, J., and Heesemann, J. (2004) A novel integrative and conjugative element (ICE) of *Escherichia coli*: the putative progenitor of the *Yersinia* high-pathogenicity island. *Mol. Microbiol.*, **51**, 837–848.

13 Ochman, H., Lawrence, J.G., and Groisman, E.A. (2000) Lateral gene transfer and the nature of bacterial innovation. *Nature*, **405**, 299–304.

14 Omelchenko, M.V., Makarova, K.S., Wolf, Y.I., Rogozin, I.B., and Koonin, E.V. (2003) Evolution of mosaic operons by horizontal gene transfer and gene displacement *in situ*. *Genome. Biol.*, **4**, R55.

15 Grozdanov, L., Raasch, C., Schulze, J., Sonnenborn, U., Gottschalk, G., Hacker, J., and Dobrindt, U. (2004) Analysis of the genome structure of the nonpathogenic probiotic *Escherichia coli* strain Nissle 1917. *J. Bacteriol.*, **186**, 5432–5441.

16 Kaper, J.B., Nataro, J.P., and Mobley, H.L. (2004) Pathogenic *Escherichia coli*. *Nat. Rev. Microbiol.*, **2**, 123–140.

17 Bergthorsson, U., and Ochman, H. (1998) Distribution of chromosome length variation in natural isolates of *Escherichia coli*. *Mol. Biol. Evol.*, **15**, 6–16.

18 Brzuszkiewicz, E., Brüggemann, H., Liesegang, H., Emmerth, M., Ölschläger, T., Nagy, G., Albermann, K., Wagner, C., Buchrieser, C., Emödy, L., Gottschalk, G., Hacker, J., and Dobrindt, U. (2006) How to become a uropathogen: comparative genomic analysis of extraintestinal pathogenic *Escherichia coli* strains. *Proc. Natl. Acad. Sci. U. S. A.*, **103**, 12879–12884.

19 Dobrindt, U. (2005) (Patho-)Genomics of *Escherichia coli*. *Int. J. Med. Microbiol.*, **295**, 357–371.

20 Gal-Mor, O., and Finlay, B.B. (2006) Pathogenicity islands: a molecular toolbox for bacterial virulence. *Cell. Microbiol.*, **8**, 1707–1719.

21 Nougayrède, J.P., Homburg, S., Taieb, F., Boury, M., Brzuszkiewicz, E., Gottschalk, G., Buchrieser, C., Hacker, J., Dobrindt, U., and Oswald, E. (2006) *Escherichia coli* induces DNA double-strand breaks in eukaryotic cells. *Science*, **313**, 848–851.

22 Ogura, Y., Ooka, T., Asadulghani, Terajima, J., Nougayrede, J.P., Kurokawa, K., Tashiro, K., Tobe, T., Nakayama, K., Kuhara, S., Oswald, E., Watanabe, H., and Hayashi, T. (2007) Extensive genomic diversity and selective conservation of virulence-determinants in enterohemorrhagic *Escherichia coli* strains of O157 and non-O157 serotypes. *Genome. Biol.*, **8**, R138.

23 Ohnishi, M., Terajima, J., Kurokawa, K., Nakayama, K., Murata, T., Tamura, K., Ogura, Y., Watanabe, H., and Hayashi, T. (2002) Genomic diversity of enterohemorrhagic *Escherichia coli* O157 revealed by whole genome PCR scanning. *Proc. Natl Acad. Sci. USA*, **99**, 17043–17048.

24 Perna, N.T., Plunkett, G., 3rd, Burland, V., Mau, B., Glasner, J.D., Rose, D.J., Mayhew, G.F., Evans, P.S., Gregor, J., Kirkpatrick, H.A., Posfai, G., Hackett, J., Klink, S., Boutin, A., Shao, Y., Miller, L., Grotbeck, E.J., Davis, N.W., Lim, A., Dimalanta, E.T., Potamousis, K.D., Apodaca, J., Anantharaman, T.S., Lin, J., Yen, G., Schwartz, D.C., Welch, R.A., and Blattner, F.R. (2001) Genome sequence of enterohaemorrhagic *Escherichia coli* O157:H7. *Nature*, **409**, 529–533.

25 Tobe, T., Beatson, S.A., Taniguchi, H., Abe, H., Bailey, C.M., Fivian, A., Younis, R., Matthews, S., Marches, O., Frankel, G., Hayashi, T., and Pallen, M.J. (2006) An extensive repertoire of type III secretion effectors in *Escherichia coli* O157 and the role of lambdoid phages in their dissemination. *Proc. Natl. Acad. Sci. USA*, **103**, 14941–14946.

26 Zhang, Y., Laing, C., Steele, M., Ziebell, K., Johnson, R., Benson, A.K., Taboada, E., and Gannon, V.P. (2007) Genome evolution in major *Escherichia coli* O157:H7 lineages. *BMC Genomics*, **8**, 121.

27 Moulin-Schouleur, M., Reperant, M., Laurent, S., Bree, A., Mignon-Grasteau, S., Germon, P., Rasschaert, D., and Schouler, C. (2007) Extraintestinal pathogenic *Escherichia coli* strains of avian and human origin: link between phylogenetic relationships and common virulence patterns. *J. Clin. Microbiol.*, **45**, 3366–3376.

28 Hejnova, J., Dobrindt, U., Nemcova, R., Rusniok, C., Bomba, A., Frangeul, L., Hacker, J., Glaser, P., Sebo, P., and Buchrieser, C. (2005) Characterization of the flexible genome complement of the commensal *Escherichia coli* strain A0 34/86 (O83: K24: H31). *Microbiology*, **151**, 385–398.

29 Rasko, D.A., Rosovitz, M.J., Myers, G.S., Mongodin, E.F., Fricke, W.F., Gajer, P., Crabtree, J., Sebaihia, M., Thomson, N.R., Chaudhuri, R., Henderson, I.R., Sperandio, V., and Ravel, J. (2008) The pangenome structure of *Escherichia coli*: comparative genomic analysis of *E. coli* commensal and pathogenic isolates. *J. Bacteriol.*, **190**, 6881–6893.

30 Grozdanov, L., Zähringer, U., Blum-Oehler, G., Brade, L., Henne, A., Knirel, Y.A., Schombel, U., Schulze, J., Sonnenborn, U., Gottschalk, G., Hacker, J., Rietschel, E.T., and Dobrindt, U. (2002) A single nucleotide exchange in the *wzy* gene is responsible for the semirough O6 lipopolysaccharide phenotype and serum sensitivity of *Escherichia coli* strain Nissle 1917. *J. Bacteriol.*, **184**, 5912–5925.

31 Janka, A., Bielaszewska, M., Dobrindt, U., Greune, L., Schmidt, M.A., and Karch, H. (2003) Cytolethal distending toxin gene cluster in enterohemorrhagic *Escherichia coli* O157:H- and O157:H7: characterization and evolutionary considerations. *Infect. Immun.*, **71**, 3634–3638.

32 Rendon, M.A., Saldana, Z., Erdem, A.L., Monteiro-Neto, V., Vazquez, A., Kaper, J.B., Puente, J.L., and Giron, J.A. (2007) Commensal and pathogenic *Escherichia coli* use a common pilus adherence factor for epithelial cell colonization. *Proc. Natl. Acad. Sci. U. S. A.*, **104**, 10637–10642.

33 Johnson, T.J., Johnson, S.J., and Nolan, L.K. (2006) Complete DNA sequence of a ColBM plasmid from avian pathogenic *Escherichia coli* suggests that it evolved from closely related ColV virulence plasmids. *J. Bacteriol.*, **188**, 5975–5983.

34 Johnson, T.J., Siek, K.E., Johnson, S.J., and Nolan, L.K. (2006) DNA sequence of a ColV plasmid and prevalence of selected plasmid-encoded virulence genes among avian *Escherichia coli* strains. *J. Bacteriol.*, **188**, 745–758.

35 Johnson, T.J., Wannemuehler, Y.M., and Nolan, L.K. (2008) Evolution of the *iss* gene in *Escherichia coli*. *Appl. Environ. Microbiol.*, **74**, 2360–2369.

36 Dobrindt, U., Blum-Oehler, G., Nagy, G., Schneider, G., Johann, A., Gottschalk, G., and Hacker, J. (2002) Genetic structure and distribution of four pathogenicity islands (PAI I(536) to PAI IV(536)) of uropathogenic *Escherichia coli* strain 536. *Infect. Immun.*, **70**, 6365–6372.

37 Guyer, D.M., Kao, J.S., and Mobley, H.L. (1998) Genomic analysis of a pathogenicity island in uropathogenic *Escherichia coli* CFT073: distribution of homologous sequences among isolates from patients with pyelonephritis, cystitis, and catheter-associated bacteriuria and from fecal samples. *Infect. Immun.*, **66**, 4411–4417.

38 Touchon, M., Hoede, C., Tenaillon, O., Barbe, V., Baeriswyl, S., Bidet, P., Bingen, E., Bonacorsi, S., Bouchier, C., Bouvet, O., Calteau, A., Chiapello, H., Clermont, O., Cruveiller, S., Danchin, A., Diard, M., Dossat, C., Karoui, M.E., Frapy, E., Garry, L., Ghigo, J.M., Gilles,

A.M., Johnson, J., Le Bouguenec, C., Lescat, M., Mangenot, S., Martinez-Jehanne, V., Matic, I., Nassif, X., Oztas, S., Petit, M.A., Pichon, C., Rouy, Z., Ruf, C.S., Schneider, D., Tourret, J., Vacherie, B., Vallenet, D., Medigue, C., Rocha, E.P., and Denamur, E. (2009) Organised genome dynamics in the *Escherichia coli* species results in highly diverse adaptive paths. *PLoS Genet.*, **5**, e1000344.

39 Hochhut, B., Wilde, C., Balling, G., Middendorf, B., Dobrindt, U., Brzuszkiewicz, E., Gottschalk, G., Carniel, E., and Hacker, J. (2006) Role of pathogenicity island-associated integrases in the genome plasticity of uropathogenic *Escherichia coli* strain 536. *Mol. Microbiol.*, **61**, 584–595.

40 Middendorf, B., Hochhut, B., Leipold, K., Dobrindt, U., Blum-Oehler, G., and Hacker, J. (2004) Instability of pathogenicity islands in uropathogenic *Escherichia coli* 536. *J. Bacteriol.*, **186**, 3086–3096.

41 Rajakumar, K., Sasakawa, C., and Adler, B. (1997) Use of a novel approach, termed island probing, identifies the *Shigella flexneri she* pathogenicity island which encodes a homolog of the immunoglobulin A protease-like family of proteins. *Infect. Immun.*, **65**, 4606–4614.

42 Turner, S.A., Luck, S.N., Sakellaris, H., Rajakumar, K., and Adler, B. (2004) Role of *attP* in integrase-mediated integration of the *Shigella* resistance locus pathogenicity island of *Shigella flexneri*. *Antimicrob. Agents Chemother.*, **48**, 1028–1031.

43 Wilde, C., Mazel, D., Hochhut, B., Middendorf, B., Le Roux, F., Carniel, E., Dobrindt, U., and Hacker, J. (2008) Delineation of the recombination sites necessary for integration of pathogenicity islands II and III into the *Escherichia coli* 536 chromosome. *Mol. Microbiol.*, **68**, 139–151.

44 Ahmed, N., Dobrindt, U., Hacker, J., and Hasnain, S.E. (2008) Genomic fluidity and pathogenic bacteria: applications in diagnostics, epidemiology and intervention. *Nat. Rev. Microbiol.*, **6**, 387–394.

45 Olesen, B., Kolmos, H.J., Ørskov, F., and Ørskov, I. (1998) *Escherichia coli* bacteraemia in patients with and without haematological malignancies: a study of strain characters and recurrent episodes. *J. Infect.*, **36**, 93–100.

46 Zdziarski, J., Svanborg, C., Wullt, B., Hacker, J., and Dobrindt, U. (2008) Molecular basis of commensalism in the urinary tract: low virulence or virulence attenuation? *Infect. Immun.*, **76**, 695–703.

47 Goerke, C., Matias, S., Papenberg, Y., Dasbach, S., Dietz, K., Ziebach, R., Kahl, B.C., and Wolz, C. (2004) Increased frequency of genomic alterations in *Staphylococcus aureus* during chronic infection is in part due to phage mobilization. *J. Infect. Dis.*, **189**, 724–734.

48 Mathee, K., Narasimhan, G., Valdes, C., Qiu, X., Matewish, J.M., Koehrsen, M., Rokas, A., Yandava, C.N., Engels, R., Zeng, E., Olavarietta, R., Doud, M., Smith, R.S., Montgomery, P., White, J.R., Godfrey, P.A., Kodira, C., Birren, B., Galagan, J.E., and Lory, S. (2008) Dynamics of *Pseudomonas aeruginosa* genome evolution. *Proc. Natl. Acad. Sci. USA*, **105**, 3100–3105.

49 Falush, D., Kraft, C., Taylor, N.S., Correa, P., Fox, J.G., Achtman, M., and Suerbaum, S. (2001) Recombination and mutation during long-term gastric colonization by *Helicobacter pylori*: estimates of clock rates, recombination size, and minimal age. *Proc. Natl. Acad. Sci. USA*, **98**, 15056–15061.

50 Gressmann, H., Linz, B., Ghai, R., Pleissner, K.P., Schlapbach, R., Yamaoka, Y., Kraft, C., Suerbaum, S., Meyer, T.F., and Achtman, M. (2005) Gain and loss of multiple genes during the evolution of *Helicobacter pylori*. *PLoS Genet.*, **1**, e43.

51 Linz, B., Balloux, F., Moodley, Y., Manica, A., Liu, H., Roumagnac, P., Falush, D., Stamer, C., Prugnolle, F., van der Merwe, S.W., Yamaoka, Y., Graham, D.Y., Perez-Trallero, E., Wadstrom, T., Suerbaum, S., and Achtman, M. (2007) An African origin for the intimate association between humans and *Helicobacter pylori*. *Nature*, **445**, 915–918.

52 Kraft, C., Stack, A., Josenhans, C., Niehus, E., Dietrich, G., Correa, P., Fox, J.G., Falush, D., and Suerbaum, S. (2006) Genomic changes during chronic *Helicobacter pylori* infection. *J. Bacteriol.*, **188**, 249–254.

53 Oh, J.D., Kling-Backhed, H., Giannakis, M., Xu, J., Fulton, R.S., Fulton, L.A., Cordum, H.S., Wang, C., Elliott, G., Edwards, J., Mardis, E.R., Engstrand, L.G., and Gordon, J.I. (2006) The complete genome sequence of a chronic atrophic gastritis *Helicobacter pylori* strain: evolution during disease progression. *Proc. Natl. Acad. Sci. USA*, **103**, 9999–10004.

2
Antimicrobial Mechanisms of Neutrophils

Catherine Chaput and Arturo Zychlinsky

2.1
Introduction

Around 1870, Paul Ehrlich developed staining techniques to study blood and bone marrow. He first established that blood cells showed affinities for alkaline, acidic and neutral dyes, discriminating between three kinds of granulated white blood cells: normoblasts, megaloblasts and leukemic cells. In 1879, Ehrlich established the neutral staining technique that was able to stain basophile leukocytes, acidophile leukocytes (also known as eosinophils) and a new group that he called 'neutrophil' leukocytes. Human neutrophils are also called polymorphonuclear leukocytes (PMN) because of the multilobulated and segmented nuclei. They are small cells, around 10 μm in diameter and contain many granules.

Neutrophils are the most abundant population of circulating white blood cells in humans (70–55% of white blood cells). These myeloid cells originate and mature in the bone marrow (Figure 2.1). Fourteen days after maturation, the neutrophil is released into the bloodstream where its lifespan is 6 to 10 h. Because of their high numbers and short lifespan, the body must produce between 10^8 to 10^{11} neutrophils/day. Mature neutrophils are the first immune cells recruited from the bloodstream to the site of inflammation (Section 2.2) where the activated neutrophils clear microbes through different processes: phagocytosis (Section 2.3), degranulation (Section 2.4) and/or after binding to Neutrophil Extracellular Traps (NETs, Section 2.5). The importance of neutrophils in inflammation is demonstrated by the severe immunodeficiency and susceptibility to infections of neutropenic patients (Section 2.6). When neutrophils are not recruited to an inflammatory site, they are eliminated by resident macrophages mainly in the liver and the spleen.

Bacterial Virulence: Basic Principles, Models and Global Approaches. Edited by Philippe Sansonetti
© 2010 WILEY-VCH Verlag GmbH & Co. KGaA, Weinheim
ISBN: 978-3-527-32326-5

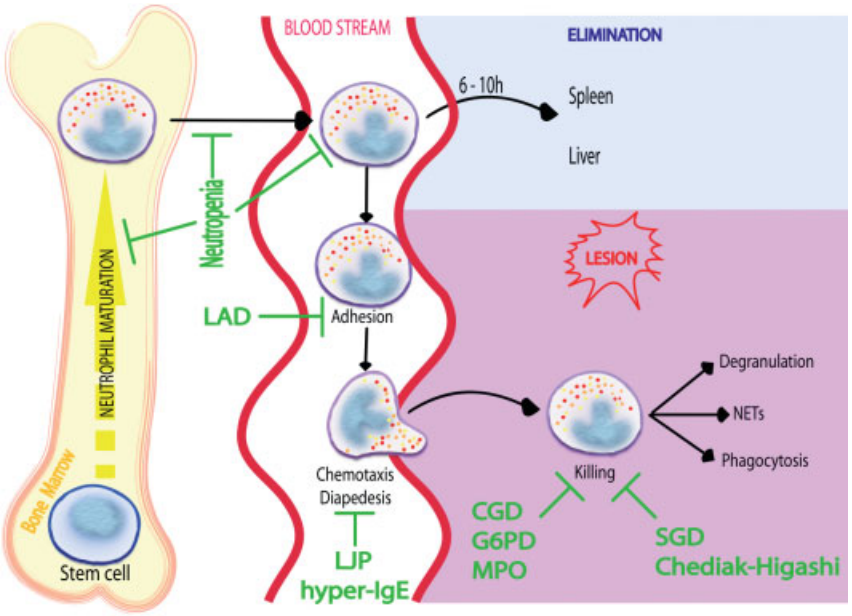


Figure 2.1 Life and disorders of neutrophils. The neutrophil matures in the bone marrow and migrates to the blood circulation. In the absence of recruitment, the neutrophil is eliminated after 6–10 h mainly in the liver and the spleen. In the case of a lesion in a tissue, the neutrophil is recruited from the bloodstream by adhesion to endothelial cells and diapedesis through the endothelium. Once at the inflammation site, the neutrophil clears microbes by phagocytosis, degranulation and/or binding to neutrophil extracellular traps (NETs). Neutropenia occurs when neutrophil maturation or migration from the bone marrow to the blood are counteracted, or when the neutrophil is eliminated due to autoantibodies against neutrophils. Other functional disorders affect the neutrophil at different stages: leukocyte adhesion deficiency (LAD), localized juvenile periodontitis (LJP), the hyperimmunoglobulin E (hyper-IgE) syndrome, Chédiak-Higashi syndrome, neutrophil-specific granule deficiency (SGD): chronic granulomatous disease (CGD), myeloperoxidase (MPO) deficiency and glucose-6-phosphate dehydrogenase (G6PD) deficiency.

2.2 Recruitment to Infection Sites

Neutrophil recruitment is a complex and multi-step process, not all stages of which are well understood (Figure 2.2). The recruitment of neutrophils requires interaction and adhesion with an activated endothelium to facilitate the entry of the neutrophil into the infected tissue. The sensing of microbes in a tissue, e.g. after injury, is the first necessary step. It occurs at postcapillary venules, high endothelial venules of lymphatic organs or atherosclerotic arteries. Contact between neutrophils and the endothelium is related to the shear stress generated by blood flow, which is at its lowest in the postcapillary venules [1].

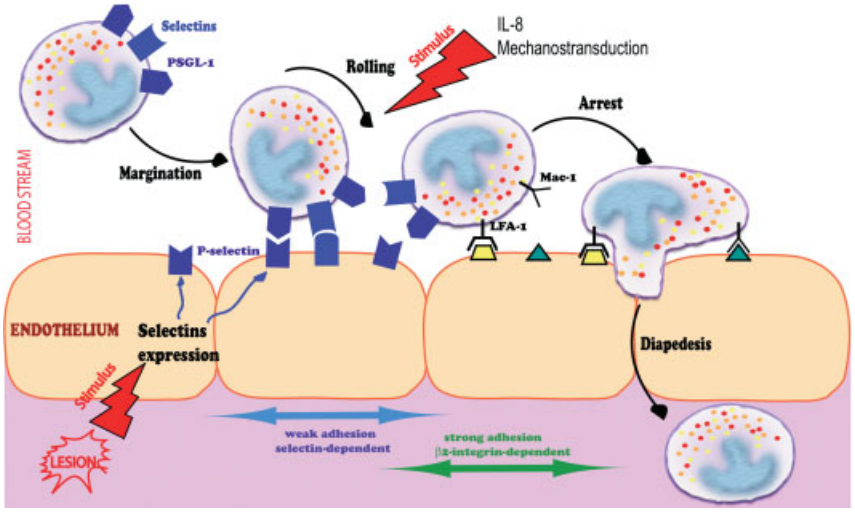


Figure 2.2 Neutrophil recruitment from the bloodstream to the site of infection. Primary neutrophil capture is mediated by adhesion to selectins. The weakness of the binding gives a phenotype called 'rolling'. After priming of the neutrophil, a stronger adhesion occurs mainly due to β2- integrins: Lymphocyte Function Antigen-1 (LFA-1) and Macrophage antigen-1 (Mac-1). The arrest of the neutrophil on the endothelial cell allows the transmigration or diapedesis through the endothelium.

After activation of the endothelium, the first contact of the neutrophil with activated endothelial cells requires at least two phenomena: (i) margination, which consists of the movement of the neutrophil from the central stream to the periphery of the vessel [2] and (ii) the expression of selectins (Table 2.1) [3]. This family of cell adhesion molecules is expressed by activated endothelial cells to allow the first contact with neutrophils. Selectins are expressed at the tips of microvilli and bind with rapid rates of association and dissociation. This selectin-dependent loose contact leads to the tethering and the rolling of the neutrophil on the endothelium [1].

The transition from leukocyte rolling to arrest is due to stronger binding to β2-integrins. β2-Integrins in a low affinity conformation are already present in the membrane of the resting neutrophils. However, mechanotransduction signaling via PSGL-1 binding of selectin(s) and stimulation of chemokines, for example with IL-8, stimulate expression of β2-integrins which mediates stronger adhesions. These β2-integrins are the Lymphocyte Function Antigen-1 (LFA-1, or CD18/CD11a) and Macrophage antigen-1 (Mac-1, or CD18/CD11b). The shift to a high affinity state and the clustering of β2-integrins leads to maximal endothelial–neutrophil adhesion and arrest the neutrophil. During this arrest, the selectins are shed [4, 5].

A second wave of stimulation is necessary for the transmigration (or diapedesis) through the epithelium and toward the infection site. While it is known that a

Table 2.1 Receptors and ligands involved in neutrophil recruitment.

Receptor	Cell	Ligand	Cell	Function
P-selectin	Endothelium	PSGL-1	Neutrophil	Rolling
L-selectin	Neutrophil	Sialylated carbohydrate (sLe^a, sLe^x)	Endothelium	Rolling
E-selectin	Endothelium	Sialylated carbohydrate (sLe^x) PSGL-1	Neutrophil	Rolling
LAF-1	Neutrophil	ICAM-1, -2 and -3	Endothelium	Firm adhesion
Mac-1	Neutrophil	ICAM-1	Endothelium	Firm adhesion
		iC3b (complement)	–	Phagocytosis opsonin-dependent
CD11c/CD18	Neutrophil	iC3b (complement)	–	Phagocytosis opsonin-dependent
CD31	Neutrophil	PECAM-1	Endothelium	Diapedesis
Fcγ receptors: FcγRIIA (CD32) FcγRIIIb (CD16) FcγRI (CD64)	Neutrophil	Fc fragment (antibodies)	–	Phagocytosis opsonin-dependent

chemoattractant gradient, mainly maintained by IL-8, is required to complete the process of transmigration, the actual mechanism remains unclear. The receptor CD31 on neutrophils recognizes PECAM-1 and it is the only protein known to be involved in this process [2]. At this point, Neutrophil Elastase (NE) is mobilized to the membrane by degranulation (see Section 2.4) and is localized to the migrating front of the cell. The exact function of NE in this process remains unclear, but it is unlikely that NE disrupts the adherent junction. Two models have been proposed for transmigration: a paracellular process consisting of neutrophil migration between endothelial cells and a transcellular diapedesis where the neutrophil moves through endothelial cells via pores. The paracellular transmigration is based on *in vitro* and *in vivo* experiments. Transcellular diapedesis has been observed *in vivo* but not *in vitro*. The existence of pores in the endothelial cell for transcellular migration might explain the speed in resealing the endothelial lining [6].

After transmigration, the neutrophil is fully activated and shows enhanced bactericidal activity. In this state the cell changes the expression of chemoattractant receptors and, consequently, chemotaxis is altered. Interestingly, at this point neutrophils become refractory to some cell death signals, for example Tumor Necrosis Factor (TNF) [2].

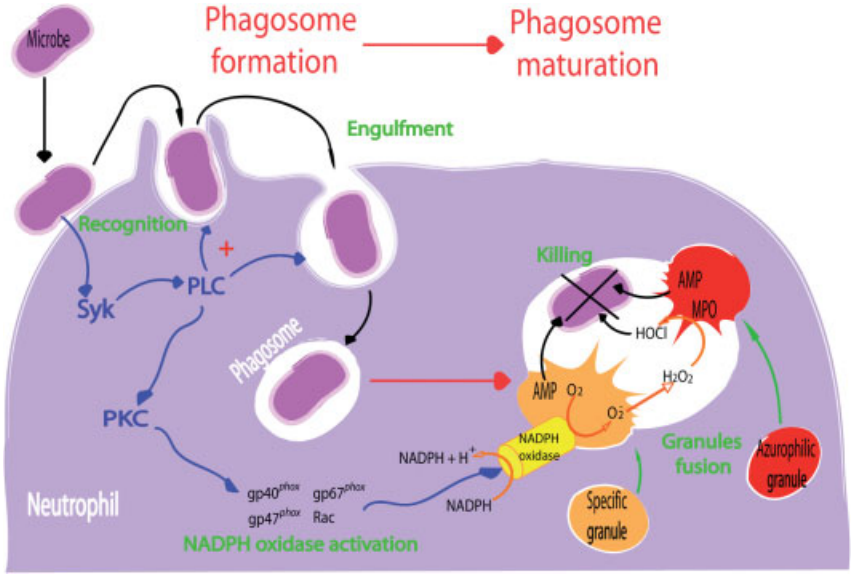


Figure 2.3 Phagocytosis by neutrophils. After recognition of a microbe, the Syk-dependent signaling cascade activates the phospholipase C (PLC) necessary for the engulfment of the microbe and triggers protein kinase C (PKC) leading to NADPH oxidase activation. Inside the neutrophil, the phagosome containing the microbe fuses with granules. The microbe is killed within the mature phagosome by reactive oxygen species produced by NADPH oxidase and MPO, and by antimicrobial peptides (AMP).

2.3 Phagocytosis

Phagocytosis is the ingestion and clearance of microbes, cells or particles bigger than 0.5 µm in diameter. This process has been studied mainly in macrophages because of the availability of cell lines and the access to genetic tools. Phagocytosis in neutrophils is similar but not identical to that in macrophages. Phagocytosis occurs in two steps. First, upon recognition of microbes, phagosomes are formed and the microbe is engulfed. During the second step, the phagosome matures and eliminates the microbe intracellularly (Figure 2.3).

Phagosome formation in neutrophils and macrophages seems to be similar at this stage. The interaction of the microbe with the neutrophil can be direct or opsonin-dependent, the latter being more efficient. Opsonization is the interaction of the microbe with the professional phagocyte via opsonins such as complement and/or antibodies. The neutrophil opsonin receptors are the β2-integrin Mac-1 for C3bi complement recognition and Fcγ receptors, FcγRIIA (or CD32), FcγRIIIb (or CD16) and FcγRI (CD64), for antibody recognition. Fcγ receptors mainly recognize the isotype IgG.

It has been well established that in macrophages the first interaction between microbes and the neutrophil initiates a complex signaling cascade through the activation of the kinase Syk. In turn, Syk activates phosphoinositide kinases and phospholipase C (PLC) which are required to finalize phagosome formation. PLC generates diacylgycerol, which triggers protein kinase C (PKC), a protein involved in the maturation but not in the formation of the phagosome. The initiation of this cascade leads to actin polymerization and membrane remodeling, which contribute to the formation of the phagosomal cup and its sealing [7].

Phagosome maturation in neutrophils is poorly studied and appears to be different than in other phagocytes. After internalization, the phagosome fuses with secretory vesicles and granules by a mechanism known as degranulation (see Section 2.4). Maturation in neutrophils consists of the acquisition of oxygen-dependent and -independent antimicrobial activities that synergize. Oxygen-independent killing depends on antimicrobial compounds that are contained in the granules (Section 2.4). The oxygen-dependent mechanism is based on the production of reactive oxygen species (ROS) in the phagosome by NADPH oxidase, a complex that is recruited into the phagosomal membrane by vesicular fusion. The current model for the assembly of the NADPH oxidase, proposes that flavocytochrome b558 which is an integral membrane complex of Nox2 (also known as $gp91^{phox}$) and $p22^{phox}$ first localize to the membrane. After the Syk-dependent activation of PKC, $p47^{phox}$ is phosphorylated allowing it to interact with $p22^{phox}$ at the membrane. When $p47^{phox}$ localizes to the membrane, the activator $p67^{phox}$ interacts with Nox2 and $p40^{phox}$. The $p40^{phox}$ subunit was shown to be part of the complex but its effect on the NADPH oxidase is unclear. Finally, the GTPase protein Rac interacts with Nox2 and then with $p67^{phox}$ [8].

Once assembled, the complex generates superoxide anion O_2^- by transferring an electron from the cytosolic NADPH to the oxygen in the lumen of the phagosome. The superoxide can interact with phagosomal targets either directly or by dismutation into hydrogen peroxide (H_2O_2). In presence of chloride, Myeloperoxidase (MPO) transforms H_2O_2 into HOCl which has strong antimicrobial activity. In the phagosome, HOCl is produced at high enough concentrations to mediate bacterial killing and modify microbial and host molecules [7].

Interestingly, while the macrophage phagosome acidifies during its maturation, it was shown that the neutrophil phagosome stays nearly neutral even though the phagosome fuses with acidic granules. The neutrality of the neutrophil phagosome is mainly due to the NADPH oxidase and MPO activities. The process of ROS production consumes a lot of protons which are internalized in the phagosome by V-ATPase and by other channel(s) [9].

2.4
Exocytosis of Secretory Vesicles and Degranulation

Degranulation consists of the exocytosis of granules which results in the delivery of their membranes and soluble contents to the extracellular compartment. The

exocytosis of secretory vesicles also plays an important role during migration and phagocytosis.

2.4.1 Granules and Secretory Vesicles

Granules are vesicles containing membranes and soluble proteins essential to the neutrophil. They store pre-synthesized antimicrobial components and/or receptors. There are three types of granules which differ in size, morphology, electron density and protein content: azurophil, specific and gelatinase granules, also known as primary, secondary and tertiary granules, respectively. Granules are made sequentially during maturation in the bone marrow. First, the azurophil granules are generated, then specific and finally the gelatinase granules. Some proteins are present in more than one type of granule while others are specific markers for a particular subset. In fact, some proteins inactivate each other and cannot coexist within the same vesicle.

Azurophilic granules are spherical or ellipsoid in shape and contain MPO. There are two subpopulations: one is poor in defensins and is made early in myeloid differentiation, while the second subpopulation is rich in defensins. Both subpopulations contain MPO, α-defensins, bactericidal/permeability-increasing protein (BPI), seprocidins and azurocidin. As mentioned earlier (Section 2.3), MPO is a peroxidase which catalyzes the production of HOCl. α-Defensins are small cationic peptides and represent 5% of the granule content. Four α-defensins are found in primary granules: human neutrophil peptide -1, -2, -3 and -4 (HNP-1, -2, -3 and -4). HNPs can kill bacteria and fungi and enveloped viruses and protozoa. The killing mechanism of HNPs is proposed through the formation of a multimeric transmembrane pore. BPI has antibacterial properties and can bind to the lipopolysaccharides of Gram-negative bacteria and also enhance phagocytosis by acting like an opsonin. Seprocodicins are serine proteases with microbicidal activity and proteolytic activity against the extracellular matrix (elastin, fibronectin, laminin, collagen type IV and vitronectin). The three main seprocodicins present in azurophil granules are proteinase-3 (PR-3), cathepsin G and neutrophil elastase (NE). NE specifically degrades the virulence factors of Gram-negative bacteria [10]. Azurocidin, a seprocodicin that is proteotically inactive, is also present in these granules.

Specific granules have irregular and elongated forms. They are large vesicles rich in antibiotic substances: NADPH oxidase, lactoferrin, neutrophil gelatinase associated lipocalin (NGAL), hCAP-18 and lysozyme. These granules carry 85% of the cellular NADPH oxidase content which is crucial for antimicrobial activity. Lactoferrin and NGAL are bacteriostatic because they chelate iron. Lactoferrin is often used as a marker for this type of granule. hCAP-18 is cleaved, extracellularly or inside the phagosome, by PR-3 (present in azurophil granules). This cleavage releases the C-terminus fragment LL-37 which has antimicrobial activity against Gram-negative and -positive bacteria. Finally, lysozyme cleaves peptidoglycan, an essential cell wall component of almost all bacteria.

Gelatinase granules are small and poor in antimicrobial substances. They are however, a reservoir of membrane receptors (e.g. Mac-1 and fMLP-receptor) and metalloproteases that are matrix-degrading enzymes (MMPs). The granular MMPs are: MMP-8 (collagenase), MMP-9 (gelatinase) and MMP-25 (leukolysin). MMPs are stored as inactive proforms that are proteolytically activated after exocytosis.

Secretory vesicles are rich in β2-integrins (CD11b/CD18), complement receptor 1 (CR1) and receptor for formylated bacterial peptides (fMLP receptors). The soluble content of these vesicles is plasmatic, indicating its endocytic origin. Upon stimulation, the secretory vesicles are mobilized to the plasma membrane and provide a new source of receptors and other functional proteins. The exocytosis of these vesicles is important during rolling on an activated endothelium to establish firm adhesion with β2-integrins [11, 12].

2.4.2 Mobilization and Fusion

Granules are spread throughout the cytoplasm and are recruited for fusion at either the plasma membrane or the phagosome. The precise mechanism of granule mobilization and delivery is not completely understood. Some key components, however, have already been described.

The elevation of cytosolic calcium is the first stimulus that triggers granule exocytosis. During phagocytosis, actin coats the phagosomal cup and rapidly disassembles after sealing. It has been proposed that actin depolymerization is regulated by the Ca^{2+} concentration to allow the docking and fusion of vesicles and granules. Interestingly, fusion of granules at the plasma membrane seems to occur near nascent phagosomes. This could explain why exocytosis at the plasma membrane is calcium-dependent. There are different Ca^{2+} thresholds for exocytosis of granules and vesicles. The Ca^{2+} concentration required for exocytosis, from the highest to the lowest is: azurophil, specific, gelatinase granules and secretory vesicles. This indicates that the first vesicles recruited are the secretory vesicles which require the smallest changes in Ca^{2+} concentration.

The soluble *N*-ethylmaleimide-sensitive-fusion-protein attachment protein receptor (SNARE) and Rab (GTPase) families of proteins are partially responsible for secretion specificity. A SNARE on a donor membrane interacts with a cognate SNARE on the target membrane to form a stable complex. This complex brings the two membranes into contact and promotes their fusion. In the activated neutrophil, SNAP23 and VAMP-2, two SNARE proteins of the specific and gelatinase granules, are translocated at the plasma membrane. Rab proteins, especially Rab5a, also confer specificity for the docking of the donor to the target membrane. Microtubules appear to be involved in the preferential delivery of granules to the phagosome.

To summarize, upon stimulation, secretory vesicles are the first to be recruited to the plasma membrane. Once fused, the exposed receptors modify the capacity

of neutrophils to respond to stimuli like chemoattractants and allow firm adhesion to the endothelial cells. Later, the neutrophil secretes matrix-degrading enzymes from gelatinase granules for the transmigration through tissue. Finally, when the neutrophil is in contact with microbes, it generates and releases antimicrobial components through the last degranulations of specific and azurophilic granules [13].

2.5 NETs

Neutrophils extracellular traps (NETs) were first reported in 2004, based mainly on microscopic observations. NETs and their formation are still poorly understood but it appears that they represent a new extracellular antimicrobial activity for neutrophils and other granulocytes [14]. NETs are composed of chromatin (DNA and histones) and granular proteins. NETs have been observed in human appendicitis, murine pneumococcal pneumonia, *Shigella*-induced experimental dysentery in rabbits and bovine mastitis.

NET formation, also known as NETosis, follows a regulated process. Upon stimulation, the neutrophils undergo at least four discrete and sequential morphological modifications. First, the multilobulated shape of the nucleus is lost, as well as the characteristics of euchromatin (decondensed DNA) and heterochromatin (condensed DNA). The nuclear envelope forms vesicles, allowing the nuclear and cytoplasmic components to mix. Third, the granules dissolve, likely permitting the interaction of granular proteins with chromatin. Finally, the cell membrane ruptures expelling the NET from the cell as it dies [15, 16].

Stimuli such as IL-8, lipopolysaccharides, bacteria, fungi, beads coated with antibodies and activated platelets induce NETosis. These factors are also potent stimuli for other neutrophil functions such as phagocytosis and chemotaxis. It is not known how environmental conditions, the state of neutrophil activation and the action(s) of microbes dictate NETosis or other functions. However, all these stimuli activate the NADPH oxidase and the production of ROS through the activation of PKC and inhibitors of ROS production block NETosis. Furthermore, neutrophils from patients with NADPH oxidase deficiencies cannot form NETs (Section 2.6). Hydrogen peroxide can compensate for a lack of NADPH oxidase activity, indicating that this enzymatic activity is responsible for initiating NETosis [15, 16].

NETs are able to trap and control the growth of bacteria and fungi (e.g. *Candida albicans*), but the antimicrobial mechanism is unclear and probably results from the synergy of different NET components [15]. Interestingly, James Hirsch, described in the late 1950s the potent antimicrobial activity of histones [17]. Other investigators confirmed Hirsch's original observations. NETs might be the biological process through which histones acn can be in contact with microbes.

2.6
Neutrophil Function Deficiencies

Patients with a reduced number or malfunction of neutrophils suffer from recurrent bacterial or fungal infections, underscoring the importance of neutrophils in the human immune defense system. The pathogens responsible for these recurrent infections are mainly opportunistic. This might reflect the type of microbes that patients are exposed to since most of those described live in America, Europe or Japan.

An abnormally reduced number of neutrophils, below 1.5×10^9 per liter of blood, is called neutropenia. Neutropenia can be inherited or acquired as a result of drug treatments, malnutrition, or infections. Interestingly, mutations in NE (ELA2) or in NE target genes are the most prevalent form of inherited neutropenia. These patients are often more susceptible to *Staphylococcus aureus* or Gram-negative bacteria [18, 19].

Neutrophil dysfunctions occur at different stages depending on the component affected. Known dysfunctions affect the binding of the neutrophil to the endothelium, chemotaxis, ingestion of microbes, degranulation and/or antimicrobial activity [20] (Figure 2.1).

2.6.1
Disorders of Adhesion and Chemotaxis

Defective adhesion and/or chemotaxis results in impaired recruitment to infection sites and neutrophil activation. There are three types of leukocyte adhesion deficiencies (LAD): LAD I, LAD II and LAD III. LAD I, the most frequent of the three, results from a genetic defect in CD18, the common chain of β2-integrins, which is required for the formation of LFA-1 (CD18/CD11a), Mac-1 (CD18/CD11b) and p150,95 (CD18/CD11c). Thus, neutrophils of LAD I patients are severely affected in adhesion, but also in complement-dependent phagocytosis mainly through Mac-1/C3bi binding β2-integrin recognition is also involved in the co-stimulatory signal for adhesion, degranulation and activation of NADPH oxidase. A CD18 deficiency affects several stages of neutrophil function. LAD I patients succumb to recurrent bacterial and fungal infections, with a high prevalence of *Staphylococcus aureus* and Gram-negative enterobacteria. LAD II and III are rare disorders. LAD II is due to a mutation in the membrane transporter for fucose, leading to a loss of expression of fucosylated glycans on the cell surface. Fucosylated glycans, like CD15s, are ligands for endothelial selectins. Fucosylated glycans, like CD15s, are ligands for endothelial selectins. LAD II patients are affected in the selectin-dependent step of neutrophil recruitment. LAD III patients are affected in the activation of multiple classes of integrins, important for the switch from low- to high-affinity β2-integrins. LAD I, II and III show similar clinical manifestations but infections are less severe in LAD II patients.

Some neutrophil disorders result in defects in chemotaxis although adhesion is normal. Non-inherited chemotaxis disorders have been reported in neonatal neu-

trophils, burn victims and in patients with bacterial sepsis or diabetes. There are two main inherited chemotaxis disorders, localized juvenile periodontitis (LJP) and hyperimmunoglobulin E (hyper-IgE) syndrome. The etiology of both disorders is unknown. LJP patients have chronic and recurrent periodontal infections and severe alveolar bone loss in the mandibule. Hyper-IgE patients have a high serum level of IgE and suffer from recurrent *Staphylococcal* infections of the skin and lung or chronic candidiasis. Interestingly, they have a deficient inflammatory response [20].

2.6.2
Disorders of Ingestion and Degranulation

Disorders of ingestion and degranulation are very rare and only two have been described: Chédiak-Higashi syndrome and neutrophil-specific granule deficiency (SGD). Chédiak-Higashi disorder is due to mutations in CHS1, a regulator of lysosomal and granule trafficking. These patients have an ineffective granulopoiesis, moderate neutropenia, and incomplete and delayed degranulation. In these patients, neutrophils, lymphocytes and natural killer cells contain giant granules. In neutrophils, giant granules result from the fusion of azurophilic and specific granules. Chédiak-Higashi patients are frequently infected by *S. aureus* (lung and skin) and develop gingivitis and periodontitis.

The SGD neutrophil, in contrast, is characterized by the absence of specific granules due to a defect in the myeloid transcription factor C/EBPε. These neutrophils are deficient in many microbicidal granular components, for example lactoferrin and defensins. SGD neutrophils have abnormal bilobed nuclei and chemotact poorly. These patients have recurrent bacterial and fungal infections that are difficult to treat, notably *S. aureus*, enteric Gram-negative bacteria, *Pseudomonas aeruginosa* and *Candida albicans* [20].

2.6.3
Disorders of Oxidative Metabolism

As mentioned in Section 2.3, oxidative metabolism in neutrophils is important for microbial killing. Patients with a defect in ROS production develop recurrent and severe infections. There are three inherited disorders of ROS production: chronic granulomatous disease (CGD), MPO and glucose-6-phosphate dehydrogenase (G6PD) deficiencies.

CGD is the most common inherited disorder of neutrophil function with clinical significance. The incidence is estimated at about 1 in 200 000 live births. CGD patients have complete, or sometimes partial, loss of NADPH oxidase activity due to genetic defects in one of the four critical subunits of the NADPH oxidase complex: Nox2 (frequency ~70%), $gp22^{phox}$ (frequency ~5%), $gp47^{phox}$ (frequency ~25%) and $gp67^{phox}$ (frequency ~5%). CGD patients are prone to infection mostly by *S. aureus*, but also, *Burkholderia cepacia*, *Serratia marscens*, *Aspergillus* species and *Nocardia*.

MPO deficiency is due to mutations in the MPO gene and is the most common inherited disorder with an estimated frequency of 1 in 4000 individuals. Interestingly, it is rarely associated with clinical symptoms, unless the patient also suffers from diabetes mellitus. In this case, the diabetic patient may develop disseminated candidiasis or other fungal infections. In this last case, the diabetic patient can develop disseminated candidiasis or other fungal infections. *In vitro*, MPO deficient neutrophils kill *C. albicans* and *Aspergillus fumigatus* hyphae poorly.

G6PD deficiency is due to mutations in G6PD, and affects mainly erythrocytes. Only in rare cases do these mutations lead to extremely low levels of G6PD in erythrocytes and neutrophils, inducing chronic and severe hemolytic anemia and CGD-like symptoms [20].

2.7 Conclusion

Neutrophils are crucial for host defense as demonstrated by the recurrent infections occuring in patients with neutrophil dysfunction. Neutrophils are the first phagocytes recruited from the bloodstream after sensing a lesion and/or infection. They switch very rapidly from resting to activated cells because they store presynthesized receptors and antimicrobial components. Once at the infected site, neutrophils clear microbes by three main mechanisms: phagocytosis, degranulation and NETs. These mechanisms are not fully understood, partially because of a lack of tools to study mature neutrophils. In the future, the development of new approaches might lead to a complete characterization of neutrophil antimicrobial mechanisms.

References

1 Simon, S.I., and Green, C.E. (2005) Molecular mechanics and dynamics of leukocyte recruitment during inflammation. *Annu. Rev. Biomed. Eng.*, **7**, 151–185.

2 Seely, A.J., Pascual, J.L., *et al.* (2003) Science review: cell membrane expression (connectivity) regulates neutrophil delivery, function and clearance. *Crit. Care*, **7** (4), 291–307.

3 Calvey, C.R., and Toledo-Pereyra, L.H. (2007) Selectin inhibitors and their proposed role in ischemia and reperfusion. *J. Invest. Surg.*, **20** (2), 71–85.

4 Ley, K. (2003) Arrest chemokines. *Microcirculation*, **10** (3–4), 289–295.

5 Zarbock, A., and Ley, K. (2008) Mechanisms and consequences of neutrophil interaction with the endothelium. *Am. J. Pathol.*, **172** (1), 1–7.

6 Kvietys, P.R., and Sandig, M. (2001) Neutrophil diapedesis: paracellular or transcellular? *News Physiol. Sci.*, **16**, 15–19.

7 Lee, W.L., Harrison, R.E., *et al.* (2003) Phagocytosis by neutrophils. *Microbes. Infect.*, **5** (14), 1299–1306.

8 Bedard, K., and Krause, K.H. (2007) The NOX family of ROS-generating NADPH oxidases: physiology and pathophysiology. *Physiol. Rev.*, **87** (1), 245–313.

9 Jankowski, A., Scott, C.C., *et al.* (2002) Determinants of the phagosomal pH in neutrophils. *J. Biol. Chem.*, **277** (8), 6059–6066.

10 Weinrauch, Y., Drujan, D., *et al.* (2002) Neutrophil elastase targets virulence factors of enterobacteria. *Nature*, **417** (6884), 91–94.

11 Borregaard, N., and Cowland, J.B. (1997) Granules of the human neutrophilic polymorphonuclear leukocyte. *Blood*, **89** (10), 3503–3521.

12 Faurschou, M., and Borregaard, N. (2003) Neutrophil granules and secretory vesicles in inflammation. *Microbes. Infect.*, **5** (14), 1317–1327.

13 Borregaard, N., Sorensen, O.E., *et al.* (2007) Neutrophil granules: a library of innate immunity proteins. *Trends Immunol.*, **28** (8), 340–345.

14 von Kockritz-Blickwede, M., Goldmann, O., *et al.* (2008) Phagocytosis-independent antimicrobial activity of mast cells by means of extracellular trap formation. *Blood*, **111** (6), 3070–3080.

15 Brinkmann, V., and Zychlinsky, A. (2007) Beneficial suicide: why neutrophils die to make NETs. *Nat. Rev. Microbiol.*, **5** (8), 577–582.

16 Fuchs, T.A., Abed, U., *et al.* (2007) Novel cell death program leads to neutrophil extracellular traps. *J. Cell. Biol.*, **176** (2), 231–241.

17 Hirsch, J.G. (1958) Bactericidal action of histone. *J. Exp. Med.*, **108** (6), 925–944.

18 Berliner, N. (2008) Lessons from congenital neutropenia: 50 years of progress in understanding myelopoiesis. *Blood*, **111** (12), 5427–5432.

19 Segel, G.B., and Halterman, J.S. (2008) Neutropenia in pediatric practice. *Pediatr. Rev.*, **29** (1), 12–23; quiz 24.

20 Dinauer, M.C. (2007) Disorders of neutrophil function: an overview. *Methods Mol. Biol.*, **412**, 489–504.

3
H. pylori Infection – The Route from Inflammation to Cancer

Tsutomu Chiba, Hiroyuki Marusawa, Hiroshi Seno, and Norihiko Watanabe

3.1
Introduction

Since the discovery of *H. pylori* in 1982, many epidemiological studies have proven an association between *H. pylori* infection and the development gastric cancer [1, 2]. Furthermore, recent retrospective and prospective studies have demonstrated that *H. pylori*-positive subjects have a significantly higher risk of gastric cancer than negative subjects [3–5]. In support of the human data, *H. pylori* infection was found to develop into gastric cancer in Mongolian gerbils [6, 7]. Thus, *H. pylori* appears to be a strong causative factor for gastric cancer.

3.2
Pathways leading to *H. pylori*-induced Gastric Carcinogenesis

There are two major pathways by which *H. pylori* infection can develop into gastric cancer: the indirect action of *H. pylori* on gastric epithelial cells causing inflammation, and the direct action of the bacteria on epithelial cells (Table 3.1). Many studies have shown the importance of gastritis in the development of gastric cancer. On the other hand, *H. pylori* can directly modulate epithelial cell function by bacterial agents such as CagA [8–10]. Both pathways appear to work in concert to promote the development of gastric cancer.

3.2.1
Indirect Action of *H. pylori* on Gastric Epithelial Cells through Induction of Gastritis

Gastric cancer is invariably associated with gastritis, and a patient's risk for gastric cancer is proportional to the severity of gastritis, particularly chronic atrophic gastritis of the corpus [11].

H. pylori-infected chronic gastritis is characterized by infiltration of a large number of CD4T cells [12]. CD4T cells are indispensable for the development of

Bacterial Virulence: Basic Principles, Models and Global Approaches. Edited by Philippe Sansonetti
© 2010 WILEY-VCH Verlag GmbH & Co. KGaA, Weinheim
ISBN: 978-3-527-32326-5

Table 3.1 Indirect and direct actions of *H. pylori* on gastric epithelial cells during gastric carcinogenesis.

I. Indirect actions
(1) Induction of innate immunity
(a) Gastric mucosa: epithelial cells and immune cells
(b) Intestinal mucosa: Peyer's patches
(2) Induction of adaptive immunity
(a) Intestinal mucosa: Peyer's patches
II. Direct actions
(1) Modulation of cellular proteins (phosphorylation etc.)
(a) Activation of SHP2
(b) Hummingbird phenotype as a result of dephosphorylation of FAK
(c) Loss of polarity resulting from binding to PAR1/MARK
(d)Induction of cell scattering caused by binding to CrK
(e)Destabilization of E-cadherin/βcatenin complex
(2) Modulation of DNA
(a) DNA methylation
(b) Gene mutation by AID

SHP2, Src homology 2-containing protein tyrosine phosphatase; FAK, focal adhesion kinase; PAR1/MARK, partitioning-defective 1/microtubule affinity-regulating kinase.

gastritis since gastritis does not develop in CD4T cell-deficient mice [13]. T cells in *H. pylori*-infected gastric mucosa produce a large concentration of IFN-γ together with a minute amount of IL-4 [12–15]. Moreover, the development of gastritis by *H. pylori* is impaired in IFN-γ-deficient mice, whereas IL-4 deficiency enhances gastritis [15]. Thus, Th1-type CD4T cells and their product, IFN-γ, appear to have important roles in the development of gastritis.

How *H. pylori* induces the Th1-type response remains unknown. We and others recently revealed that Peyer's patches in the small intestine play a crucial role in the production of *H. pylori*-specific CD4T cells, their migration into the gastric mucosa and the development of gastritis [16, 17]. It was found that in the absence of Peyer's patches *Helicobacter* infection failed to lead to gastritis despite significant colonization in the gastric mucosa. Moreover, although transfer of splenic T cells of *Helicobacter*-infected mice to *Helicobacter*-infected Rag2 knockout mice induced severe gastritis, splenic T cells from *Helicobacter*-infected Peyer's patch-deficient mice failed to produce gastritis in Rag2 knockout mice in spite of the presence of a large number of *Helicobacter* in the gastric mucosa. These data clearly show the importance of Peyer's patches and also the adaptive immunity against *Helicobacter* in the development of gastritis.

In addition to infiltration of various inflammatory cells, *H. pylori*-induced gastritis is characterized by enhanced production of a variety of cytokines in the gastric mucosa, and several cytokines are suggested to play roles in the development of cancer. Among them, many investigators have focused on interleukin-1β(IL-β) as

the factor that links gastritis and gastric cancer. El-Omar *et al*. [18] first reported an association between an IL-1β gene polymorphism and a risk of gastric cancer by *H. pylori* infection. A recent study supporting their data, demonstrated the development of gastric cancer in IL1β transgenic mice [19]. However, the data from Asian countries, including our own, do not confirm their data, suggesting that the importance of IL1β in the development of gastric cancer varies among different ethnic groups [20–22].

In addition to IL1β, production of other cytokines is also enhanced in the gastritis mucosa [23], and of note, in addition to IL1β, TNFα enhances NFκB activation. Recently, there has been much interest in the importance of the roles of NFκB in inflammation-associated carcinogenesis [24]. Several possible roles for NFκB are being considered in the development of gastric cancer. NFκB in the epithelial cells may exert an anti-apoptotic action. NFκB activation in the immune cells may accelerate gastric inflammation by enhancing the production of various cytokine. Moreover, NFκB activation in epithelial, inflammatory and mesenchymal cells may enhance COX2 production. Finally, our recent finding suggested the involvement of NFκB in *H. pylori*-induced mutagenesis [25].

Levels of IL-6 and IL-11 are also increased in the *H. pylori*-induced gastritis mucosa. Giraud *et al*. demonstrated the development of gastric cancer as a result of constitutive activation of STAT3 in mice, suggesting the importance of IL-6 and/or IL-11 signaling in the development of gastric cancer [26, 27]. Indeed, STAT3 activation accelerates nuclear localization of β-catenin [28]. We found that *H. pylori* infection induces RegIα production, a potent growth factor for gastric mucosa, in the gastric epithelial cells [29]. Interestingly, RegIα production in the gastric mucosal cells is not stimulated directly by *H. pylori* but by IL-6 in a STAT3-dependent manner [30]. Thus, IL-6/IL-11-STAT3 signaling appears to accelerate epithelial cell growth not only directly but also indirectly by stimulating RegIα production in the epithelial cells. We also showed that in addition to IL-6, RegIα production is enhanced by IFNγ, a representative Th1 cytokine [31]. These cytokines appear to be involved in the development of gastric cancer in a coordinated manner.

Innate immunity is an indispensable step not only for the generation of adaptive Th1 response against *H. pylori* infection but also for the production of various cytokines and chemokines from both immune cells and epithelial cells. Indeed, a recent study demonstrated that *H. pylori*-derived peptidoglycan is delivered to epithelial cells via a type IV secretion apparatus, and activates NFκB by binding to an intracellular pathogen-recognition molecule, NOD1 [32], leading to the production of various cytokines. Moreover, Rad *et al*. showed that bone marrow-derived dendritic cells from MyD88-deficient mice produced reduced levels of cytokines upon stimulation with *H. pylori*, suggesting that some components of *H. pylori* may stimulate Toll-like receptors [33]. Furthermore, recent studies have shown that VacA directly modulates immune cell functions by various mechanisms [34, 35].

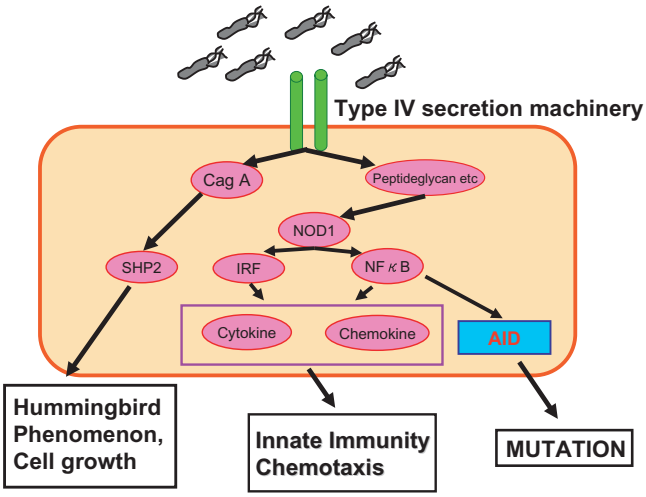


Figure 3.1 *H. pylori*-induced intracellular signaling events that are involved in gastric cancer development. AID, activation-induced cytidine deaminase; NFκB, nuclear factor-κB; SHP2, Src homology 2-containing protein tyrosine phosphatase.

3.2.2 Direct Action of *H. pylori* on Gastric Epithelial Cells

In addition to the indirect pathway of induction through inflammation, *H. pylori* promotes gastric carcinogenesis by acting directly on gastric epithelial cells (Figure 3.1). Indeed, *H. pylori* infection or the introduction of various *H. pylori* genes into the gastric epithelial cells modulates cellular growth, apoptosis, or cell migration, and changes cell shape. *H. pylori* or products of *H. pylori* may act on cellular membranes, and some of the effects may be mediated by cell surface receptors such as Toll-like receptors (TLRs). However, several studies have shown that TLRs are not important in epithelial cell responses to *H. pylori*. Instead, recent studies have demonstrated that a bacterial type IV secretion apparatus, encoded by the *H. pylori cag* pathogenicity island (*cagPAI*), plays an essential role in mediating many of the direct actions of *H. pylori* on epithelial cells by delivering bacterial agents into the cells. Indeed, CagA protein exerts its action on epithelial cells by entering the cells through this structure [36, 37].

3.2.2.1 Roles of CagA in Gastric Carcinogenesis

H. pylori has been subdivided into *cagA*-positive and -negative strains. The *cagA*-positive strains are more potent in the development of gastritis than the *cagA*-negative strains [38]. Indeed, clinical studies have demonstrated a strong association between infection with *cagA*-positive *H. pylori* and the development of gastric cancer [39, 40]. Moreover, gastric cancer patients are almost invariably infected with *cagA*-positive strains.

As described previously, CagA is delivered into gastric epithelial cells by the bacterial type IV secretion apparatus, where it undergoes tyrosine phosphorylation by Src family kinases or Abl kinase at the EPIYA motifs [8, 37, 41]. Subsequently, CagA binds to and activates Src homology 2-containing protein tyrosine phosphatase (SHP2) [10, 37], and the CagA-deregulated SHP2 exerts various actions such as activation of the Erk MAP kinase cascade [10], inhibition of focal adhesion kinase (FAK) with the resulting induction of the hummingbird phenomenon [42], and inhibition of Src family kinases by activating the C-terminal Src kinase (Csk) generating a feedback regulation loop. CagA also binds to Crk to induce cell scattering [43]. In contrast to the events involving tyrosine phosphorylation, CagA influences Grb2 and c-Met in a phosphorylation-independent manner [44, 45]. Moreover, CagA has been shown to activate the nuclear factor of activated T cells (NFAT) via stimulation of calcineurin [46]. More recently, CagA has been shown to disturb cell adhesion independently of CagA phosphorylation by disrupting tight junctions as a result of the inhibition of the kinase activity of partitioning-defective 1 (PAR1)/microtubule affinity-regulating kinase (MARK) [47]. CagA also destabilizes the E-cadherin–β-catenin complex with the resulting activation of β-catenin [48]. All of these actions of CagA may contribute to the development of gastric cancer by providing epithelial cells with a suitable environment for neoplastic transformation.

However, it should be noted that almost all *CagA*-positive *H. pylori* also contain *cagPAI*. Importantly, the functional significance of *cagA* and *cagPAI* is different. Indeed, in addition to CagA, other bacterial factors such as peptidoglycan are introduced into the host cells via the type IV secretion apparatus which is composed of the gene products encoded by *cagPAI* [32]. Thus, it is possible that some of the actions of *cagA*/*cagPAI*-positive *H. pylori* are not exerted by CagA but by some factors that are introduced into cells through the type IV secretion apparatus which is the product of *cagPAI*. Indeed, although CagA translocation and its phosphorylation are required for the appearance of the hummingbird phenomenon, these events do not play major roles in NFκB activation in epithelial cells, and it has been reported that *H. pylori*-derived peptidoglycan delivered through the type IV secretion apparatus is mainly responsible for NFκB activation [32].

Another point of interest is that many of the data relating to the direct actions of CagA were obtained from *in vitro* studies in which the *cagA* gene was transfected into epithelial cells, but not by *H. pylori* infection. Thus, the possibility remains that some of the direct actions of CagA on epithelial cells as seen in the gene transfection experiments are not physiological, and thus have been overestimated. Accordingly, the roles of CagA should be examined by *H. pylori* infection *in vitro*. In this regard, further discussion is required to determine whether or not the development of cancer as observed in *CagA* transgenic mice represents a physiological phenomenon [49].

3.2.2.2 Induction of Gene Mutations by *H. pylori*

Cancer development is characterized by the accumulation of various gene modulations including mutations. A recent study reported the presence of approximately

15 non-synonymous mutations in cancer-related genes in human colon cancers as well as breast cancers [50]. Previous clinical studies have shown that mutations of *TP53* are already present in *H. pylori*-infected chronic gastritis mucosa [51, 52]. Moreover, animal experiments using Big-Blue mice revealed a high mutation frequency in *H. pylori*-infected gastric mucosa [53]. Thus, *H. pylori* infection appears to induce gene mutations in gastric mucosal cells. It is possible that *H. pylori*-induced gastritis may enhance gene mutations by producing mutagens such as radical oxygen species (ROS). However, whether *H. pylori* directly affects epithelial cells to induce gene mutations is still unknown. Recently, we demonstrated that *H. pylori* directly induces gene mutation by enhancing expression of activation-induced cytidine deaminase (AID) in the gastric mucosal cells [25]. AID, a member of the cytidine-deaminase family that acts as an editor of DNA and RNA, is essential for somatic hypermutation and class-switch recombination of immunoglobulin genes in B lymphocytes [54]. Importantly, AID is expressed exclusively in B cells under physiological conditions. We first found that constitutive expression of AID in transgenic mice resulted in the development of gastric cancer with high mutation frequencies, suggesting that AID played a role in gastric carcinogenesis by inducing gene mutations. Surprisingly, we also found that AID was ectopically expressed in *H. pylori*-induced gastritis mucosa as well as gastric cancer tissues in humans and eradication of *H. pylori* reduced its expression. Moreover, we showed that in the gastric mucosa of *H. plori*-infected subjects a significant level of AID expression was associated with frequent *TP53* mutations, whereas in the gastric mucosa of *H. pylori*-negative subjects low or no expression of AID was accompanied by low or no *TP53* mutation frequency. Furthermore, *H. pylori* infection *in vitro* elicited ectopic expression of AID in association with a high mutation frequency of the *TP53* gene in human gastric cancer cells [25]. AID expression in B cells is stimulated via an NFκB-dependent pathway [55]. Therefore, we also examined the NFκB dependency of *H. pylori*-induced AID expression and found that *H. pylori*-induced AID expression in the gastric epithelial cells is also mediated by NFκB activation. Indeed, AID expression in gastric epithelial cells is elicited only by *cagPAI*-positive *H. pylori* that can activate NFκB. Finally, we showed that *H. pylori*-induced *TP53* mutation *in vitro* could be inhibited by blocking AID using AID siRNA. Taken together, these data strongly suggested that *H. pylori* directly induces gene mutations in epithelial cells by enhancing AID expression as a result of NFκB activation in a *cagPAI*-dependent manner (Figure 3.1). Since NFκB is activated by cytokines such as IL1β and TNFα [56], AID expression also appears to be induced by these cytokines in the gastritis mucosa. Thus, our study proposes a novel role for NFκB in inflammation-associated carcinogenesis.

It is noteworthy that the most prevalent base substitution observed in human gastric cancer is a cytidine(C) to thymidine (T) transition [50, 57], and AID theoretically induces a C to T (G to A) transition. Interestingly, however, although the frequency of C to T (G to A) transitions was highest in our *in vitro* experiments, other mutations are also produced by *H. pylori* infection as well as by the introduction of *AID*. Although the reason for this unexpected observation is unknown, the

involvement of a repair system associated with several error-prone DNA polymerases of low fidelity may be a possibility [58]. Alternatively, this phenomenon might have occurred because we used cancer cells instead of normal cells.

3.2.2.3 Induction of Aberrant DNA Methylation by *H. pylori*

In addition to the induction of gene mutations, recent studies have suggested that *H. pylori* infection enhances aberrant DNA methylation in gastric mucosa and that such methylation may participate in gastric carcinogenesis by silencing the tumor suppressor genes. Ushijima *et al.* demonstrated enhanced DNA methylation in *H. pylori*-infected non-cancerous gastritis mucosa [59]. Interestingly, they found that *H. pylori* infection resulted in DNA methylation of the genes common in Mongolian gerbils and in humans, suggesting gene specificity for aberrant DNA methylation by *H. pylori* infection (personal communication). Recently, Chan *et al.* [60] reported that polymorphisms of IL-1β are associated with CpG island methylation of *CDH1* in gastric cancer. Moreover, *H. pylori* infection or IL-1β has been shown to induce methylation of *CDH1* in a human gastric cancer cell line [61]. However, it is still unclear whether the aberrant DNA methylation results from the direct action of *H. pylori* on epithelial cells or from *H. pylori*-induced inflammation.

3.2.3 Relationship between Direct and Indirect Actions of *H. pylori* on Epithelial Cells in the Development of Gastric Cancer

At an early stage of gastritis, when a large number of *H. pylori* is present, the bacteria have both direct and indirect effects on epithelial cells. *H. pylori* accelerates gastritis not only by presenting bacterial antigens at Peyer's patches of the intestine but also by stimulating the innate immunity within the stomach. At the same time, *H. pylori* acts directly on gastric epithelial cells to induce gene mutations and enhance cell growth, to inhibit or promote apoptosis, and to modulate cell adhesion and migration by bacterial agents. It should be emphasized that the direct actions are probably targeted to the stem cells or to the progenitor cells in the isthmus of the gastric gland, because it would be difficult for the differentiated cells to be transformed into cancer cells. Thus, a large number of *H. pylori* may be required to exert direct effects on undifferentiated cells located at the isthmus. On the other hand, at a late stage of gastritis, the number of *H. pylori* decreases with progression of atrophic gastritis. At this stage, the activity of gastritis becomes less severe but the continuous supply of *H. pylori*-specific T cells from the intestine may persist despite the decrease in the number of organisms, thus contributing to the continuation of chronic gastritis. In contrast, with the decrease in the number of *H. pylori* in the gastric mucosa, it is likely that the direct influence of *H. pylori* on epithelial cells is reduced, and consequently the signal transduction events in the epithelial cells evoked directly by *H. pylori* may be inhibited. Thus, at a late stage of *H. pylori* infection, the direct actions of *H. pylori* on epithelial cells do not appear to be important. However, once the cells, stem cells or progenitor cells, acquire gene mutations as a result of the action of AID at an active

stage of gastritis, the mutated cells persist until this stage and eventually become malignant.

References

1 Parsonnet, J., Friedman, G.D., Vandersteen, D.P., *et al.* (1991) *Helicobacter pylori* infection and risk of gastric carcinoma. *N. Engl. J. Med.*, **325**, 1127–1131.

2 Nomura, A., Stemmerman, G.N., Chyou, P.H., Kato, I., Perez-Perez, G.I., and Blaser, M.J. (1991) *Helicobacter pylori* infection and gastric cancer among Japanese Americans ain Hawaii. *N. Engl. J. Med.*, **325**, 1132–1136.

3 Yamagata, H., Kiyohara, Y., Aoyagi, K., *et al.* (2000) Impact of *Helicobacter pylori* infection on gastric cancer incidence in a general Japanese population: the Hisayama study. *Arch. Intern. Med.*, **160**, 1962–1968.

4 Uemura, N., Okamoto, S., Yamamoto, S., *et al.* (2001) *Helicobacter pylori* infection and the development of gastric cancer. *N. Engl. J. Med.*, **345**, 784–789.

5 Fukase, K., Kato, M., Kikuchi, S., *et al.* (2008) Effect of eradication of *Helicobacter pylori* on incidence of metachronous gastric carcinoma after endoscopic resection of early gastric cancer: an open-labeled, randomized controlled trial. *Lancet*, **372**, 392–397.

6 Watanabe, T., Tada, M., Nagai, H., Sasaki, S., and Nakao, M. (1998) *Helicobacter pylori* infection induces gastric cancer in Mongolian gerbils. *Gastroenterology*, **115**, 642–648.

7 Hirayama, F., Takagi, S., Iwao, E., *et al.* (1999) Development of poorly differentiated adenocarcinoma and carcinoid due to long-term *Helicobacter pylori* colonization in Mongolian gerbils. *J. Gastroenterol.*, **34**, 450–454.

8 Segal, E.D., Cha, J., Lo, J., Falkow, S., and Tomkins, L.S. (1999) Altered states: Involvement of phosphorylated CagA in the induction of host cellular growth changes by *Helicobacter pylori*. *Proc. Natl. Acad. Sci. USA*, **96**, 14559–14564.

9 Asahi, M., Azuma, T., Ito, S., *et al.* (2000) *Helicobacter pylori* CagA protein can be tyrosine phosphorylated in gastric epithelial cells. *J. Exp. Med.*, **191**, 593–602.

10 Higashi, H., Nakaya, A., Tsutsumi, R., *et al.* (2004) *Helicobacter pylori* CagA induces Ras-independent morphogenic response through SHP2 recruitment and activation. *J. Biol. Chem.*, **279**, 17205–17216.

11 Chiba, T., Seno, H., Marusawa, H., Wakatsuki, Y., and Okazaki, K. (2006) Host factors are important in determining clinical outcomes of *Helicobacter pylori* infection. *J. Gastroenterol.*, **41**, 1–9.

12 Itoh, T., Wakatsuki, Y., Yoshida, M., *et al.* (1999) The vast majority of gastric T cells are polarized to produce T helper 1 type cytokines upon antigenic stimulation despite absence of *Helicobacter pylori* infection. *J. Gastroenterol.*, **34**, 560–570.

13 Eaton, K.A., Mefford, M., and Thevenot, T. (2001) The role T cell subsets and cytokines in the pathogenesis of *Helicobacter pylori* gastritis in mice. *J. Immunol.*, **166**, 7456–7461.

14 Itoh, T., Seno, H., Kita, T., Chiba, T., and Wakatsuki, Y. (2005) The response to *Helicobacter pylori* differs between patients with gastric ulcer and duodenal ulcer. *Scand. J. Gastroenterol.*, **40**, 641–647.

15 Smythies, L.E., Waites, K.B., Lindsey, J.R., *et al.* (2000) *Helicobacter pylori*-induced mucosal inflammation is Th1 mediated and exacerbated in IL4 but not IFN-gamma gene-deficient mice. *J. Immunol.*, **165**, 1022–1029.

16 Kiriya, K., Watanabe, N., Nishio, A., *et al.* (2007) Essential role of Peyer's patches in the development of Helicobacter-induced gastritis. *Int. Immunol.*, **19**, 435–446.

17 Nagai, S., Mimuro, H., Yamada, T., *et al.* (2007) Role of Peyer's patches in the induction of *Helicobacter pylori*-induced

gastritis. *Proc. Natl. Acad. Sci. USA*, **104**, 8971–8976.
18 El-Omar, E.M., Carrington, M., Chow, W.H., *et al.* (2000) Interleukin-1 polymorphisms associated with increased risk of gastric cancer. *Nature*, **404**, 398–402.
19 Tu, S., Cui, G., Takaishi, S., *et al.* (2007) Overexpression of IL-1B induced gastric inflammation and carcinoma through dysfunction of immunity and change of gastric microenvironment in transgenic mice. *Gastroenterology*, **132**, A31.
20 Seno, H., Satoh, K., Tsuji, S., *et al.* (2007) Novel IL4 and IL1RN variations associated with non-cardiac gastric cancer in Japan: comprehensive analysis of 207 polymorphisms of 11 cytokines. *J. Gastroenterol. Hepatol.*, **22**, 729–737.
21 Chen, A., Li, C.N., Hsu, P.I., *et al.* (2004) Risks of interleukin 1 genetic polymorphisms and *Helicobacter pylori* infection in the development of gastric cancer. *Aliment. Pharmacol. Ther.*, **20**, 203–211.
22 Chang, Y.W., Jang, J.Y., Kim, N.H., *et al.* (2005) Interleukin 1B cytokine in Korean patients with gastric cancer. *Int. J. Cancer*, **114**, 465–471.
23 Ohana, M., Okazaki, K., Oshma, C., *et al.* (2001) A critical role for IL7R signaling in the development of *Helicobacter felis*-induced gastritis in mice. *Gastroenterology*, **121**, 329–336.
24 Plkarsky, E., Porat, R.M., Stein, H., *et al.* (2004) NFkB functions as a tumour promoter in inflammation-associated cancer. *Nature*, **431**, 461–466.
25 Matsumoto, Y., Marusawa, H., Kinoshita, K., *et al.* (2007) *Helicobacter pylori* infection triggers aberrant expression of activation-induced cytidine deaminase in gastric epithelium. *Nat. Med.*, **13**, 470–476.
26 Judd, L.M., Alderman, B.M., Howlett, M., *et al.* (2004) Gastric cancer development in mice lacking the SHP2 binding site on the IL6 family coreceptor gp130. *Gastroenterology*, **126**, 196–207.
27 Jackson, C.B., Judd, L.M., Menheniott, T.R., *et al.* (2007) Augmented gp130-mediated cytokine signaling accompanies human gastric oncogenesis. *J. Pathol.*, **213**, 140–151.
28 Kawada, M., Seno, H., Sawabu, T., *et al.* (2006) STAT3 activation is involved in nuclear accumulation of beta-catenin in colorectal cancer. *Cancer Res.*, **66**, 2913–2917.
29 Fukui, H., Franceschi, F., Penland, R.L., *et al.* (2003) Effects of *Helicobacter pylori* infection on the link between regenerating gene expression and serum gastrin levels in Mongolian gerbils. *Lab. Invest.*, **83**, 1777–1786.
30 Sekikawa, A., Fukui, H., Fujii, S., *et al.* (2005) REG Iα protein may function as a trophic and/or anti-apoptotic factor in the development of gastric cancer. *Gastroenterology*, **128**, 642–653.
31 Sekikawa, A., Fukui, H., Fujii, S., *et al.* (2008) REG Iα protein mediates an anti-apoptitic effect of STAT3 signalling in gastric cancer cells. *Carcinogenesis*, **29**, 76–83.
32 Viala, J., Chaput, C., Boneca, I.G., *et al.* (2004) Nod1 responds to peptidoglycan delivered by the *Helicobacter pylori* cag pathogenicity island. *Nat. Immunol.*, **5**, 1166–1174.
33 Rad, R., Brenner, L., Krug, A., *et al.* (2007) Toll-like receptor-dependent activation of antigen-presenting cells affects adaptive immunity to *Helicobacter pylori*. *Gastroenterology*, **133**, 150–163.
34 Torres, V.J., VanCompernolle, S.E., Sundrud, M.S., *et al.* (2007) *Helicobacter pylori* vacuolating cytotoxin inhibits activation-induced proliferation of human T and B lymphocyte subsets. *J. Immunol.*, **179**, 5433–5440.
35 Sewald, X., Gebert-Vogl, B., Prassl, S., *et al.* (2008) Integrin subunit CD18 is the T lymphocyte receptor for the *Helicobacter pylori* vacuolating cytotoxin. *Cell Host Microbe*, **3**, 20–29.
36 Covacci, A., and Rappuoli, R. (1998) *Helicobacter pylori*: molecular evolution of a bacterial quasispecies. *Curr. Opin. Microbiol.*, **1**, 760–770.
37 Odenbreit, S., Puls, J.,Sedlmaier, B., Geriand, E., *et al.* (2000) Translocation of *Helicobacter pylori* CagA into gastric epithelial cells by type IV secretion. *Science*, **287**, 1497–1500.
38 Peek, R.M., Jr., Miller, G.G., Tham, K.T., *et al.* (1995) Heightened inflammatory response and cytokine expression *in vivo*

to cagA + *Helicobacter pylori* strains. *Lab. Invest.*, **73**, 760–770.

39 Parsonet, J., Friedman, G.D., Orentreich, N., *et al.* (1997) Risk for gastric cancer in people with CagA positive or CagA negative *Helicobacter pylori* infection. *Gut*, **40**, 297–301.

40 Blaser, M.J., Perez-Perez, G.I., Kleanthous, H., *et al.* (1995) Infection with *Helicobacter pylori* strains possessing cagA is associated with an increased risk of developing adenocarcinoma of the stomach. *Cancer Res.*, **55**, 2111–2115.

41 Tammer, I., Brandt, S., Hartig, R., *et al.* (2007) Activation of Abl by *Helicobacter pylori*: a novel kinase for CagA and crucial mediator of host cell scattering. *Gastroenterology*, **132**, 1309–1319.

42 Tsutsumi, R., Takahashi, A., Azuma, T., *et al.* (2006) Focal adhesion kinase is a substrate and downstream effector of SHP2 complexed with *Helicobacter pylori* CagA. *Mol. Cell. Biol.*, **26**, 261–276.

43 Suzuki, M., Mimuro, H., Suzuki, T., *et al.* (2005) Interaction of CagA with Crk plays an important role in *Helicobacter pylori*-induced loss of gastric epithelial cell adhesion. *J. Exp. Med.*, **202**, 1235–1247.

44 Mimura, H., Suzuki, T., Tanaka, J., *et al.* (2002) Grb2 is a key mediator of *Helicobacter pylori* CagA protein activities. *Mol. Cell*, **10**, 745–755.

45 Churin, Y., Al-Ghoul, L., Kepp, O., *et al.* (2003) *Helicobacter pylori* CagA protein targets the c-Met receptor and enhances the motogenic response. *J. Cell Biol.*, **161**, 249–255.

46 Yokoyama, K., Higashi, H., Ishikawa, S., *et al.* (2005) Functional antagonism between *Helicobacter pylori* CagA and vacuolating toxin VacA in control of the NFAT signaling pathway in gastric epithelial cells. *Proc. Natl. Acad. Sci. USA*, **102**, 9661–9666.

47 Saadat, I., Higashi, H., Obuse, C., *et al.* (2007) *Helicobacter pylori* CagA targets PAR1/MARK kinase to disrupt epithelial cell polarity. *Nature*, **447**, 330–333.

48 Franco, A.T., Israel, D.A., Washington, M.K., *et al.* (2005) Activation of β-catenin by carcinogenic *Helicobacter pylori*. *Proc. Natl. Acad. Sci. USA*, **102**, 10646–10651.

49 Ohnishi, N., Yuasa, H., Tanaka, S., *et al.* (2008) Transgenic expression of *Helicobacter pylori* CagA induces gastrointestinal and hematopoietic neoplasms in mouse. *Proc. Natl. Acad. Sci. USA*, **105**, 1003–1008.

50 Sjoblom, T., Jones, S., Wood, L.D., *et al.* (2006) The consensus coding sequences of human breast and colorectal cancers. *Science*, **314**, 268–274.

51 Morgan, C., Jenkins, G.J., Ashton, T., *et al.* (2003) Detection of p53 mutations in precancerous gastric tissues. *Br. J. Cancer*, **89**, 1314–1319.

52 Kodama, M., Murakami, K., Nishizono, A., and Fujioka, T. (2004) Animal models for the study of Helicobacter-induced gastric carcinoma. *J. Infect. Chemother.*, **10**, 316–325.

53 Touati, E., Michel, V., Thiberge, M.V., *et al.* (2003) High gastric mutant frequency in *H. pylori*-infected mice. *Gastroenterology*, **124**, 1408–1419.

54 Honjo, T., Kinoshita, K., and Muramatsu, M. (2002) Molecular mechanism of class switch recombination: linkage with somatic hypermutation. *Annu. Rev. Immunol.*, **20**, 165–196.

55 Dedeoglu, F., Horwitz, B., Chaudhuri, J., *et al.* (2004) Induction of activation-induced cytidine deaminase gene expression by IL4 and CD40 ligation is dependent on STAT6 and NFκB. *Int. Immunol.*, **16**, 395–404.

56 Endo, Y., Marusawa, H., Kinoshita, K., *et al.* (2007) Expression of human activation-induced cytidine deaminase in human hepatocytes via NF-kB signaling. *Oncogene*, **26**, 5587–5595.

57 Greenman, C., Stephens, P., Smith, R., *et al.* (2007) Patterns of somatic mutation in human cancer genomes. *Nature*, **446**, 153–158.

58 Liu, M., Duke, J.L., Richter, D.J., *et al.* (2008) Two levels of protection for the B cell genome during somatic hypermutation. *Nature*, **451**, 841–845.

59 Ushijima, T., Nakajima, T., and Maekita, T. (2006) DNA methylation as a marker

for the past and future. *J. Gastroenterol.*, **41**, 401–407.

60 Chan, A.O., Chu, K.M., Huang, C., *et al.* (2007) Association between *Helicobacter pylori* infection and interleukin 1β polymorphism predispose to CpG island methylation in gastric cancer. *Gut*, **56**, 595–597.

61 Qian, X., Huang, C., Cho, C.H., *et al.* (2008) E-cadherin promoter hypermethylation induced by interleukin-1β treatment or *H. pylori* infection in human gastric cancer cell lines. *Cancer Lett.*, **263**, 107–113.

Part II
Models

Bacterial Virulence: Basic Principles, Models and Global Approaches. Edited by Philippe Sansonetti
© 2010 WILEY-VCH Verlag GmbH & Co. KGaA, Weinheim
ISBN: 978-3-527-32326-5

4
Host–Pathogen Relationship in Skin and Soft Tissue Infections caused by Group A *streptococcus* and *Staphylococcus aureus*

Inbal Mishalian, Miriam Ravins, Moshe Baruch, Merav Persky, Ilia Belotserkovsky, and Emanuel Hanski

4.1
Introduction

Group A streptococcus (GAS) and *Staphylococcus aureus* (SA) are prominent human pathogens causing a wide variety of diseases ranging from mild skin and throat infections to life-threatening diseases. Each year worldwide, GAS is responsible for more than 600 million cases of pharyngitis and skin infections and 650 000 highly invasive infections [1]. GAS necrotizing fasciitis (NF) and toxic shock syndrome (TSS) result in case fatality rates ranging from 14 to 36%, despite prompt antibiotic treatment and surgical intervention [2]. SA is responsible for the vast majority of skin and soft tissue infections in humans, such as impetigo, folliculitis/furunculosis, and cellulitis, which result in 11.6 million outpatients and emergency room visits and 464 000 hospital admissions per year in the United States alone [3]. Although SA infections usually originate in the skin, invasive and frequently life-threatening infections are common sequelae, and include lymphangitis, septic arthritis, abscesses of various organs, osteomyelitis, bacteremia, pneumonia, meningitis, endocarditis, and sepsis [4–6]. In contrast to GAS which remains sensitive to penicillins, SA has become increasingly resistant to β-lactam antibiotics, and methicillin-resistant SA (MRSA) is currently a leading cause of hospital-acquired (nosocomial) infections. While these infections typically occur in individuals with predisposing risk factors, such as surgery, community-acquired (CA)-MRSA causes disease even in healthy individuals, and there has been an alarming increase in the number of CA-MRSA infections worldwide [7]. An estimated 18 650 deaths were associated with invasive MRSA infections during 2005 in the United States alone [8].

What makes GAS and SA such successful human pathogens capable of existing as harmless commensals, and at the same time causing diseases ranging from confined and mild infections to those that are systemic and life-threatening infections? Answers to these questions are starting to emerge. Both SA and GAS have developed many sophisticated strategies to impede innate immune system

Bacterial Virulence: Basic Principles, Models and Global Approaches. Edited by Philippe Sansonetti
© 2010 WILEY-VCH Verlag GmbH & Co. KGaA, Weinheim
ISBN: 978-3-527-32326-5

functions at multiple junctures [9–15]. The innate immunity system is designed to sense invading microorganisms and instantaneously trigger defensive reactions that counteract invading pathogens. This prompt response prevents pathogen dissemination beyond both cutaneous and mucosal epithelial barriers [16–18]. To pass the cutaneous barrier pathogens need to overcome hundreds of peptides/proteins, which have potent microbicidal activities at low concentrations. The vast arsenal of antimicrobial peptides contains defensins and cathelicidins which are key players in cutaneous immunity [19]. Once pathogens have breached the skin barrier, clearance of the invaders depends primarily on cells known as 'professional phagocytes' [20, 21]. This includes resident macrophages and recruited cells such as polymorphonuclear neutrophils (PMNs) and monocytes. Although they are not considered to be 'professional phagocytes' cell populations such as dendritic and endothelial cells can also naturally express phagocytic functions against microorganisms [22]. Leukocyte recruitment into inflamed tissue proceeds in a cascade-like fashion [23–25]. On reaching the bacteria at the site of infection PMNs are capable of microbial killing by oxygen-dependent and -independent mechanisms [26, 27]. Circulating monocytes give rise to mature tissue resident macrophages throughout the body as well as specialized cells such as dendritic cells and osteoclasts [22]. Impressive recent progress has been made with regard to the characterization of discrete subsets of monocytes and their functional characterization in the innate immune response [28]. The relative contributions of resident macrophages and the above-described monocyte subsets to the elimination of invading microorganisms and their mode of cooperation with PMNs during various infectious processes [29] are just starting to emerge.

This chapter is designed to compare the strategies used by GAS and SA to hamper host innate immunity during skin and soft-tissue infections; in particular how these pathogens evade elimination by PMNs. Although many SA and GAS infections originating in the skin may become invasive, only a few studies have been conducted in the relevant cutaneous models. In fact, most studies have been carried out either *in vitro*, *ex vivo* or in systemic infection models. Because they are more pertinent to the natural course of GAS and SA infections, the former studies are more comprehensively discussed in this review. Nonetheless we also refer to the latter type of study which is expected to have a major impact on GAS and SA pathogeneses.

4.2
Restriction of GAS and SA Cutaneous Infections by Cationic Antimicrobial Peptides and Bacterial Resistance Mechanisms to these Compounds

The human skin acts as the first line of defense against invading bacteria. It gives physical protection partly because it is composed of tightly associated epithelial cells covered by a highly cross-linked layer of keratin that is normally impenetrable to bacteria. Additionally, the skin displays microbicidal activity via an array of bioactive molecules, among which antimicrobial peptides are of critical importance to host

defenses [30–32]. In general, most of these peptides have both cationic and amphiphatic properties [33, 34] and are termed CAMPs (**c**ationic **a**nti**m**icrobial **p**eptides). These properties enable CAMPs to initially interact with anionic head groups of the microbial membrane lipids, integrate into the hydrophobic core of the membrane, and finally cause bacterial membrane disruption. Some CAMPs are also able to promote bacterial lysis by directly affecting bacterial division [35]. The production of the human and mouse cathelicidin family of antimicrobial polypeptides (LL-37 and CRAMP, respectively) in the skin occurs constitutively at a low level. Upon skin injury or during the initial steps of the infectious process there is a dramatic increase in cathelicidins in the skin which is even further increased during inflammation due to their production by recruited PMNs and activated skin keratinocytes [36]. The first unambiguous evidence that CRAMP plays an important role in restricting GAS spread from the skin into deeper tissues was found using CRAMP-deficient mice. These mice developed more rapidly-growing lesions of increased size with significantly higher bacterial counts compared to their wild type (WT) littermates when infected subcutaneously with WT GAS [37]. Furthermore, when WT mice were infected with CRAMP-resistant GAS mutants, they developed larger, more long-lasting lesions compared to mice infected with the parental GAS strain. Overall these findings clearly show that in mice CRAMP protects the skin from invasive GAS infections [37]. The role of CAMPs in preventing SA skin infections is supported by the finding that keratinocytes kill the bacteria by depositing CAMP β-defensin-3 (HBD-3) onto their surface during contact with SA at the apical layers of the human epidermis [38]. The fact that individuals with atopic dermatitis, who suffer from recurrent SA skin infections, are defective in the ability to mobilize bactericidal concentrations of HBD-3 onto the bacterial surface provides further evidence for this mechanism [39].

The importance of CAMPs in the innate immune skin defenses against SA and GAS is also highlighted by the multiple countermeasures that these pathogens have developed to limit the effectiveness of CAMPs [40, 41]. This includes a reduction of the net negative charge of SA and GAS cell envelopes [42–46], cleavage of CAMPs by secreted [47] or surface bound [48] bacterial proteases, and inactivation of CAMPs upon binding to secreted bacterial proteins [49, 50]. A fascinating mechanism that has been recently discovered is that by lowering its hydrophobicity by overproduction of a hydrophilic surface protein, SA can render itself resistant to bactericidal human skin fatty acids and peptides [51].

4.3
Leukocytes and Cutaneous SA and GAS Infection

4.3.1
Polymorphonuclear Neutrophils (PMNs)

Once SA and GAS succeed in breaching the local defenses of the skin they then face phagocytes which muster to ingest, kill and digest bacteria within minutes.

Of these cells PMNs are the most numerous and best equipped for these missions. The PMN response is considered to constitute the first line of systemic defenses against invading GAS and SA and is a critical determinant in the outcome of these infections [15, 52]. This is best manifested in neutropenic patients or individuals with congenital or acquired defects in PMN function who suffer from recurrent invasive infections caused by these pathogens [53, 54].

The ability of SA and GAS to evade human host defenses and invade from the skin into deeper tissues causing life-threatening systemic infections is well known, but the underlying mechanisms have been elusive. As early as 1967 Agarwal reported that 'highly virulent' but not 'non-virulent' SA clinical isolates were able to stall the development of inflammation at the site of infection when injected subcutaneously [55]. This finding was subsequently reproduced using cell-wall fractions [56] and in 1976 was shown to result from the active inhibition of PMNs chemotaxis [57]. This apparently provides SA with a window of time for proliferation and subsequent dissemination.

A paucity of PMN infiltration of the skin and subcutaneous tissues has also been reported in cases of human GAS NF [58, 59]. In the baboon model of human NF, surviving baboons showed an intense PMN influx into the site of inoculation, whereas those who died had no PMN influx at all [60]. In the murine model of human NF, mice challenged with a WT GAS strain developed a lethal infection that was typified by the absence of PMN migration to the initial site of infection, mirroring the pathological findings in the NF patient from whom the bacterium was first isolated. Furthermore, in this patient PMN infiltration was absent in necrotic tissues with a high bacterial load but was clearly apparent in the non-necrotic surrounding tissues that were free of bacteria [61]. These findings suggest that GAS can effectively retard the influx of PMNs to the site of infection. Finally, a retrospective histopathological analysis of soft tissues debrided from human GAS NF patients revealed a strong correlation between the severity of the disease and a paucity or absence of PMNs at the infection site [62].

4.3.1.1 The Interactions of SA and GAS with Innate Immunity leading to PMN Response

Innate immune responses are initiated by pattern recognition receptors (PRRs), among which Toll-like receptors (TLRs) play a major role [16, 17]. TLR2 has been reported to be involved in the recognition of several SA components including alanylated LTA and lipopeptides (for a review see [10]). Indeed, mice deficient in either TLR2 or MyD88, an adapter molecule mediating TLR signaling [63], were more susceptible to systemic SA infection than WT mice [64, 65]. Using a cutaneous model of SA infection, Miller *et al.* [66] demonstrated that while TLR-2- and MyD88-deficient mice developed larger skin lesions compared to WT mice, only MyD88 null mice showed also a higher persistent bacterial load due to a defect in PMN recruitment, and hence a failure in bacterial eradication. This defect was caused by impaired production of the murine CXCL2 and CXCL1 chemokines (granulocyte chemotactic protein 2, MIP-2 and keratinocyte-derived chemokine KC, respectively) [66]. Taken together, these findings suggested that a receptor

other than TLR2 (which also signals via the MyD88 adapter) is responsible for PMN recruitment via induction of large amounts of MIP-2 and KC. In fact, it was discovered that IL-1R which utilizes the MyD88 adapter for signaling [63], exhibits a diminished ability to clear cutaneous SA infections. Like MyD88-deficient mice, IL-1R null mice developed large lesions with persistent high SA counts, impaired PMN influx due to diminished production of KC and MIP-2, and consequently a failure to eradicate the bacteria [66]. Since PMN-depleted mice are highly susceptible to cutaneous challenge with SA [52], these findings strongly support the notion that IL-1R signaling is crucial for mobilization of PMNs in SA skin infections. More recently it was shown that bone marrow (BM)-derived cells produce IL-1β that signals via IL-1R which is present on the skin of resident non-BM-derived cells. This triggers a high production of KC and MIP-2, leading to PMN recruitment and resulting in the resolution of SA cutaneous infections [67]. The importance of IL-1R signaling in the containment of SA infections in humans was recently demonstrated by the findings that nine children with inherited MyD88 deficiency, which were normally resistant to most common bacteria, viruses, fungi and parasites, were vulnerable to a narrow range of pyogenic bacterial pathogens including SA [68].

Dendritic cells were shown to play a role in the innate immune response to cutaneous GAS infections [69]. When infected with GAS *ex-vivo* these cells produced the cytokines interleukins 12 and 6 (IL-12, IL-6) and tumor necrosis factor α (TNF-α) in a MyD88-dependent manner. It was found that multiple TLRs rather than a single TLR trigger the production of these pro-inflammatory cytokines [70]. Using bone marrow-derived mouse macrophages it was recently shown that GAS induces the production of pro-inflammatory cytokines such as IL-6 and TNF-α in MyD88-mediated signaling processes [71]. However, the induction was independent of TLR2, TLR4 and TLR9 as well as IL-1 receptor signaling. It was also demonstrated that GAS elicits MyD88-independent signaling that results in type I interferon production [71]. Whether or not recruitment of PMNs to the site of cutaneous GAS infection is mediated via stimulation of a single or multiple innate immunity receptors and requires the MyD88 adapter remains to be determined.

SA and GAS skin infections were recently reported to cause severe pulmonary damage as a result of a strong inflammatory response. The surface-bound protein A of SA, which impedes SA phagocytosis by PMNs, was shown to also act directly as an immune effector. Protein A binds and activates TNF-α receptor 1 (TNFR1) [72]. This induces mobilization of TNFR1 to the apical surface of the airway epithelium and an increased production of the CXCL8 chemokine (also known as interleukin 8, IL-8) by NF-κB activation [73, 74]. As a result, a massive recruitment of PMNs occurs, causing lung damage due to uncontrolled PMN degranulation. Protein A and TNFR1 interaction is thus essential for the development of SA pneumonia since TNFR1 null mice were not susceptible to SA pneumonia and a protein A-defective mutant of SA did not cause infection in WT mice [72].

The GAS M-protein was shown to cause vascular leakage and lung damage due to its immunomodulatory activities [75]. It is one of the best characterized virulence factors that prevents opsonophagocytosis of GAS in whole blood. M protein

is usually covalently anchored to the GAS cell wall but can be released from the surface by bacterial and host proteases. It forms complexes with fibrinogen (Fb) that result in the accumulation of blood cells and protein aggregates in the lung tissue. M-Fb complexes bind to β_2-integrins on PMNs, causing degranulation and release of a protein known as HBP, **h**eparin-**b**inding **p**rotein. The released HBP augments the local inflammation due to its binding to β_2-integrins on the surfaces of PMNs and monocytes. In response to HBP, monocytes generate pro-inflammatory cytokines which further intensify lung damage [76].

4.3.1.2 Avoidance of SA and GAS Clearance by PMNs

The various strategies employed by GAS and SA to avoid PMN recruitment, opsonization and subsequent clearance have been discussed in several excellent recent reviews [9, 12–15, 77–80]. Here, we will elaborate on studies that are mostly relevant to skin and soft-tissue infections and discuss newly discovered bacterial strategies.

Evasion of PMN Migration In response to chemotactic signals, circulating PMNs begin to tether to and roll on endothelial cells at the luminal side. They then form firm adhesions as a result of chemokine activation processes, extravasate and finally migrate to the site of infection [24, 81]. The initial chemotactic signals include N-formyl peptides that originate from either rupture of host cells causing the release of mitochondrial proteins or from proteins of invading bacterial pathogens [82]. The other signals are exclusively host derived and include the complement anaphylatoxin C5a and chemokines [81]. In general, the strategy that GAS utilizes to impede PMN recruitment to the infection site is to inactivate chemotactic signals, while the main tactic SA uses is to block PMN receptors involved in chemotactic signal sensing and receptors required for emigration from the endothelium, followed by migration to the site of infection.

All three pathways of the complement system converge at the end of their enzymatic cascades to generate C5a and the terminal membrane attack complex [83]. C5a is the most potent pro-inflammatory mediator produced in the complement cascade. Binding of C5a to its receptor induces a range of inflammatory effects including a strong chemoattraction for PMNs, monocytes and macrophages, upregulation of leukocyte adhesion molecule expression, activation of PMNs to increase the uptake and killing of microorganisms, increase in vasodilatation, modulation of leukocytes and endothelial cells pro-inflammatory cytokine expression, reduction in PMN apoptosis, and activation of the coagulation pathway [83].

The GAS streptococcal C5a peptidase (ScpA) is a surface-associated subtilisin-like serine protease [84, 85]. ScpA inactivates C5a by generating a single cleavage in the C-terminal region of C5a and releasing a heptapeptide [86] that is essential for the binding of C5a to its receptors [87]. The virulence capacity of ScpA was attributed to its soluble form which is shed from the GAS surface either by an autocatalytic cleavage [88] or by the GAS cysteine protease, SpeB [89]. Recently, however, it was demonstrated that the surface exposed glyceraldehyde-3-phosphate dehydrogenase binds C5a, and this facilitates its cleavage by the surface-bound

ScpA [90]. Although it is widely accepted that ScpA is a key virulence factor of GAS, a mutant deficient of ScpA was as lethal as the parental WT strain in a soft-tissue air sac model [91]. Nevertheless, a greater number of PMNs infiltrated air sacs of mice infected with a ScpA mutant compared to those infected with the WT strain at 4 h after inoculation, suggesting that ScpA may perhaps play a role in very early stages of GAS infection [91]. However, no difference in mice survival, PMN recruitment, bacterial counts or size of cutaneous lesions was observed for WT and a ScpA-deficient mutant in a murine model of human soft-tissue infection [92]. This apparent failure of ScpA to affect GAS virulence could be attributed to the fact that ScpA has far lower activity against murine C5a than the human C5a [93]. The generation of humanized C5a transgenic mice should reveal whether or not ScpA fulfills a central role in the pathogenesis of cutaneous GAS infections.

Despite the fact that ScpA plays a minor role in preventing PMN migration in a murine model of human soft-tissue infection, a paucity of PMNs was observed in necrotic infected fascia [61], suggesting that GAS possesses additional effective mechanisms to impede PMN migration to the site of cutaneous infections. IL-8 plays a central role in the formation of firm adhesions of PMNs with endothelial cells, in PMN activation, extravasation, and finally in their migration along the infected tissues [23, 25]. These properties make IL-8 an excellent target for subverting PMN functions by pathogens. Indeed, it was discovered that in GAS culture medium there is serine-dependent proteolytic activity that degrades IL-8 *in vitro* and also during GAS infection of cultured epithelial cells [61]. This soluble IL-8 proteolytic activity was subsequently identified as belonging to a GAS surface protein termed SpyCEP i.e. *S. pyogenes* cell envelope protease [94]. The degradation of IL-8 was a result of a single specific cleavage in the C-terminus between a glutamine at position 59 and an arginine at position 60, generating 6- and 2-kDa polypeptides [94]. This cleavage obliterated the ability of IL-8 to both recruit and activate PMNs as determined *in vitro* using a partially purified protease isolated from the GAS culture medium [94]. The fact that the protease also cleaved the IL-8 functional murine homologues, MIP-2 and KC [92], suggested that it would be possible to assess its contribution to virulence using the murine model of human soft-tissue infection. Indeed, by applying a set of isogenic mutants it was established that the IL-8 protease plays a major role in the pathogenesis of GAS soft-tissue infections [92]. Because the protease belongs to the family of subtilisin-like proteases, displaying a high amino acid homology with ScpA but having a strict specificity for CXC chemokines, it was named ScpC, for-streptococcal CXC chemokine protease [92]. When inoculated subcutaneously, WT GAS caused large necrotic lesions characterized by low PMN infiltration and a high bacterial load, which subsequently led to a lethal systemic infection (see Figure 4.1). In contrast, a mutant deficient of ScpC caused smaller lesions and higher PMN infiltration (see Figure 4.1), resulting in bacterial clearance and mouse survival. In addition, soft-tissue biopsies obtained during the infectious process from mice inoculated with the WT strain displayed MIP-2 and KC levels that were significantly lower compared to the levels of these chemokines detected in biopsies from mice inoculated with the ScpC mutant [92]. Moreover, systemic depletion of PMNs or

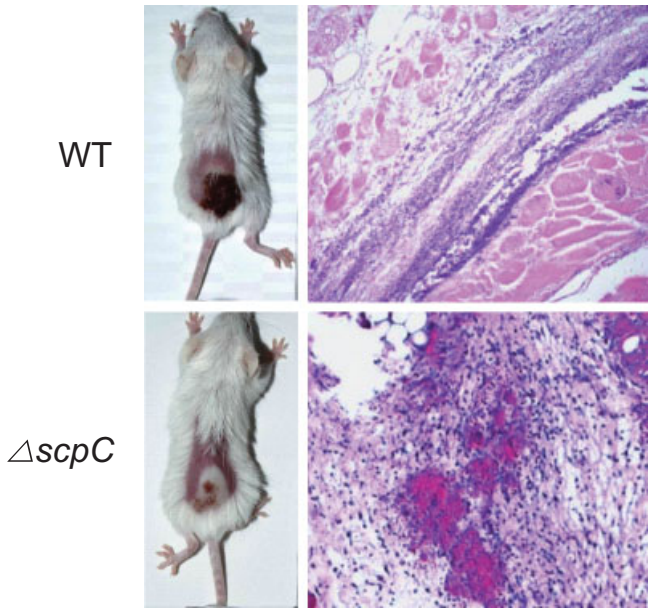


Figure 4.1 Macroscopic and microscopic images of mice challenged with a WT and a Δ*scpC* mutant. BALB/c mice were inoculated subcutaneously with 10^8 colony-forming units of WT GAS M14 strain JS95 (upper panels) or JS95-derived Δ*scpC* mutant (lower panels). Two days after the challenge, photographs and biopsies of two representative mice were taken and histophathology examinations were performed. The mouse that has been injected with the WT displays a markedly larger and more necrotic skin lesion than the mouse injected with the Δ*scpC* mutant. The hematoxylin and eosin staining of the biopsy taken from the mouse injected with JS95 shows an extensive necrosis of the subcutaneous fat tissue and of the fascia, a large bacterial load and paucity of PMN infiltration. In contrast, the biopsy from the mouse injected with the Δ*scpC* mutant reveals massive PMN recruitment to the infection site with a much lower bacterial load and a lower level of necrosis.

blockage of mouse CXCR2/IL-8R by administration of anti-mouse CXCR2 antibody rendered the ScpC mutant fully virulent, proving that ScpC mainly acts by degrading host CXC chemokines, resulting in diminished PMN recruitment to the cutaneous site of infection [92]. More recent studies have demonstrated that ScpC also cleaves the CXCL6 granulocytes chemotactic protein 2 (GCP-2) and the CXCL1 growth related oncogene alpha (GROα), diminishing expression of the integrin β2-subunit CD11b on PMNs, and hence inhibiting superoxide generation in response to N-formyl peptides [95]. Most recently, it was shown that ScpC promotes a polymicrobial necrotizing skin infection by *in-trans* retarding PMN recruitment and bacterial clearance. Necrotic skin lesions produced by a mixed infection of mice with WT GAS and vancomycin-resistant *Enterococcus faecalis* (VRE) were significantly larger than those produced by a mixture of a ScpC mutant and VRE. In addition, the WT strain but not the ScpC mutant promoted VRE survival [96]. Furthermore, ScpC significantly reduces GAS killing by isolated

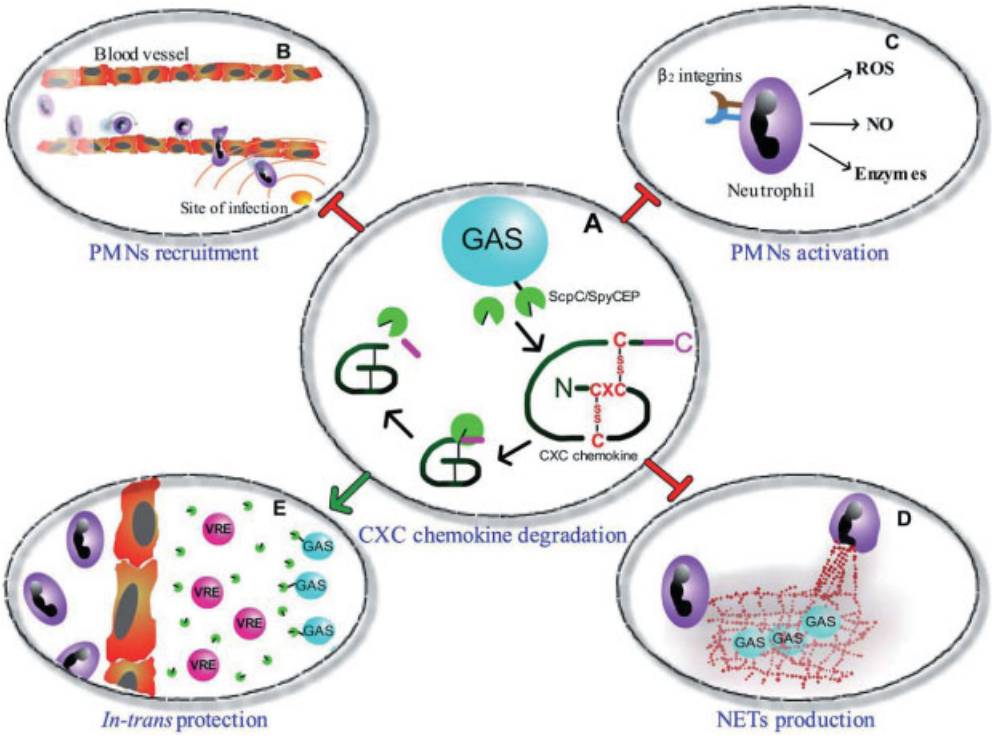


Figure 4.2 A schematic model depicting several mechanisms by which ScpC contributes to GAS virulence. ScpC cleaves CXC chemokines at the C-terminal rendering them incapable of interacting with their cognate CXC receptors (A). Degradation of the CXC chemokines inhibits essential steps in PMN chemotaxis, including endothelial emigration and migration towards the site of infection (B). The cleavage of CXC chemokines inhibits PMN priming, namely appearance of β_2-integrin receptors on PMN surface. This prevents PMN activation as reflected in the production of reactive oxygen and nitrogen species and the production of hydrolytic enzymes (C). IL-8 degradation reduces NETs production [96] (D). ScpC released from GAS surface acts *in-trans* promoting survival of vancomycin-resistant *Enterococcus faecalis* (VRE) in a mixed cutaneous infection with GAS by all the activities described above (E).

PMNs either directly, most probably by preventing PMNs priming for activation [94–96], or indirectly by reducing NETs (neutrophil extracellular traps) production (see below and reference [96]). The different modes by which ScpC contributes to GAS pathogenesis in soft-tissue infections are illustrated in Figure 4.2.

The SA extracellular adherence protein (Eap) is a highly cationic secreted protein which is able to rebind to the SA surface. Through its capacity to bind a broad spectrum of host proteins, Eap probably mediates bacterial adhesion to host cells [98, 99]. Isolated Eap can bind to the endothelial intercellular adhesion molecule (ICAM-1) and as a result inhibits the interaction of the latter with PMN β_2 integrins (CDR3 and lymphocyte function antigen LFA-1) [100]. In addition, Eap blocks PMN adhesion to fibrinogen [100]. Since the interactions of β_2 integrins with

ICAM-1 and with fibrinogen are important for PMN emigration from the circulation as well as migration along the infected tissue, Eap was expected to have a pronounced effect on PMN recruitment to SA infection sites. In fact, a SA strain producing Eap was found to induce significantly lower PMN recruitment in a mouse peritonitis model compared to that of an Eap-deficient isogenic mutant [100]. Using a mouse wound-healing model, the WT SA strain producing Eap but not its isogenic Eap-deficient mutant was able to delay wound closure by impaired infiltration of PMNs and macrophages [101].

SA's CHIPS (chemotaxis inhibitory protein of staphylococci) is one of the best studied anti-chemotactic factors. This 14.1-kDa secreted protein binds to two chemotactic receptors, the C5aR and the formylated peptide receptor (FPR) [102, 103]. While the binding domain of CHIPS to FPR has been localized to reside on the extreme N-terminus of the protein, the localization of the binding domain to C5aR has not been precisely mapped, but is distal to the FPR-binding domain and located on the C-terminal portion of CHIPS [104]. The binding of CHIPS to C5aR and FPR receptors inhibits their signal transduction processes as reflected by the impairment of PMN chemotaxis towards C5a and formylated peptides, as well as in the diminished mobilization of intracellular calcium [102, 103]. Most importantly, although CHIPS affinity to mouse C5aR is about 30-fold lower than its affinity to the human receptor, intravenous injection of CHIPS into mice inhibited PMN migration when mice were subsequently challenged with a peritoneal injection of C5a [102]. Furthermore, sera of SA-infected patients contain antibodies that prevent binding of CHIPS to C5aR, enhancing the likelihood that CHIPS plays an important role in the pathogenesis of human SA infections [105].

Interference of GAS and SA with PMN killing Although it has been known for more than a century that the surface M-protein and the hyaluronic acid capsule impair GAS opsonophagocytic killing [106, 107], the fact that M-protein and the capsule are key virulence factors in soft-tissue infection was established only a decade ago [108]. This is mainly because of advances in constructing non-polar isogenic mutants and the use of murine models reproducing important features of human infections [108].

Historically, GAS resistance to phagocytosis was determined by incubating GAS in non-immune whole human blood and comparing the bacterial colony forming units before and after incubation. By expressing M-protein on their surface, GAS strains limit the deposition of complement component C3b, thus successfully proliferating in non-immune whole blood. By contrast, M-protein-deficient mutants become circumferentially covered with C3b and are readily killed [109, 110]. The low levels of C3b present on the surface of M-protein-producing GAS strains, together with the fact that C3b is further degraded to iC3b, which displays a substantially lower affinity than C3b for the CR1 receptor [111], has led to the widely-accepted view that M-protein prevents GAS from forming a firm association with PMNs and subsequent uptake. Activation of PMNs by the addition of C5a to whole blood up-regulates the expression of functional CR3 receptors, which exhibit a high affinity towards the surface-bound iC3b [112], thus leading to

increased association of GAS with activated PMNs and consequently to uptake [113]. Similarly, GAS growth in C5a-treated whole blood was slower than growth in non-treated blood due to some degree of PMN-mediated bacterial killing [113]. In soft-tissue infections, GAS faces activated PMNs. Thus rather than by preventing GAS uptake, we assume that M-protein probably contributes to evasion of bacterial clearance either through decreased formation of C5a due to inhibition of complement activation and hence impaired PMN influx, or through enhanced survival of GAS within PMNs.

In spite of the widely-accepted view that the M-protein prevents uptake of GAS by PMNs, carefully carried out analyses using flow cytometry and fluorescent and electron microscopy clearly demonstrated that human PMNs take up M-protein-deficient and -proficient GAS strains with similar efficiency. However, while the former were rapidly killed, the latter survived and multiplied within PMNs [114]. In a subsequent study it was shown that M-protein expression inhibited the fusion of azurophilic granules with phagosomes, thus promoting GAS intracellular survival [115]. The intracellular GAS maintained infectivity as demonstrated by gentamicin-treated PMNs isolated from human blood.

Gene expression microarray analyses revealed that the transcription of genes associated with cell wall synthesis, oxidative stress response, virulence, and transcriptional regulation was significantly increased after 3 h of GAS interactions with PMNs. During this time the rate of GAS killing by PMNs progressively decreased and reached its lowest constant value, suggesting that GAS undergoes a process of adaptation and develops resistance to intracellular killing by PMNs [116]. For this purpose GAS required a two-component system (TCS), Ihk/Irr, which exhibited increased expression during the interaction with PMNs [116, 117]. It was discovered that Ihk/Irr also mediates PMN lysis [116]. Compared to the parental WT, a mutant defective in *irr* produced smaller skin lesions and lower bacteremia in murine models of human soft-tissue and systemic infection, respectively [117]. These cumulative data suggested that a fitness response is triggered in GAS to accommodate the environmental conditions existing inside PMNs. Indeed, it was found that GAS expression of GpoA, the major glutathione peroxidase, allows GAS to adapt to oxidative stress [118], and is essential for GAS pathogenesis in a murine model of soft-tissue infection [119]. Thus the induced fitness of GAS to cope with the conditions that exist within PMNs increases its ability to resist phagocytic killing and subsequently cause PMN lysis [116, 117, 119].

Inasmuch as the PMN response is highly beneficial to the host, its timely termination is critical for limiting collateral tissue damage [120]. Consequently, apoptosis is triggered in PMNs upon microbial uptake and is mainly aimed at resolving the infection and maintaining PMN homeostasis [120]. In contrast to several pathogens including SA, GAS is capable of altering normal phagocytosis-induced PMN apoptosis, thus eliciting an accelerated process which leads to PMN necrosis [121]. In summary, not only is GAS able to escape killing by PMNs, it also meddles with major PMN physiological programs for its own benefit. Whether or not specific virulence factors are involved in these modes of operation of GAS avoidance of clearance by PMNs remains to be determined.

Recently, a fascinating new mode by which PMNs eliminate pathogens was discovered. Following activation, PMNs undergo a distinct cell death program causing them to release extracellular structures consisting of a DNA backbone with embedded antimicrobial peptides and enzymes known as NETs (neutrophil extracellular traps). NETs capture and kill a wide range of Gram-positive and -negative bacteria independently of phagocytic uptake [122–124]. Therefore it is not surprising that virulence mechanisms have evolved to counteract NETs. A GAS triple-mutant strain deficient of chromosome and prophage-encoded extracellular DNases was less virulent than the parental WT strain in a systemic mouse model [125]. In a murine model of soft-tissue infection the triple mutant produced smaller and less necrotic lesions than the corresponding WT, and was more sensitive to extracellular killing by PMNs, strongly suggesting that GAS DNases might act by dissipating NETs [125]. Indeed, using live-cell imaging it was demonstrated that expression of DNase from a plasmid in a GAS strain producing a low level of extracellular DNase activity or in the non-pathogenic *Lactococcus lactis*, endowed these bacteria with the ability to degrade NETs *in vitro* and in a mouse model of human soft-tissue infection [126]. As IL-8 is a key factor in induction of NET formation [97], elimination of IL-8 by ScpC significantly contributes to the prevention of NET development and hence to impairment of NET-mediated extracellular bacterial killing (see Figure 4.2 and [96])

While GAS's complement inhibitory strategy is to recruit and exploit host regulators, the SA strategy is to secrete proteins that directly inhibit the complement cascade. Among these proteins, staphylococcus complement inhibitors (SCINs) are the most effective because they inhibit the activity of C3 convertases which control all complement actions. Consequently, this minimizes the initial interactions of SA with PMNs [127–129]. Nonetheless, as early as 1895, Van de Velde reported studies suggesting that staphylococci are taken up and survive within PMNs [130]. Survival of SA within PMNs was confirmed in several *in-vitro* studies published in the 1960s [131–133]. Forty years later it was shown that survival required the global virulence regulator Sar, since a *sar*-deficient mutant was more sensitive to PMN killing and the bacteria were present in tight phagosomes as opposed to WT SA which formed spacious phagosomes and some bacteria were even present in the cytoplasm [134]. More importantly, like GAS [135, 136] intracellular SA maintained infectivity and PMNs containing intracellular SA established infection in naive animals [134]. As SA strains producing low levels of catalase (KatA) were more sensitive to killing by PMNs, it was assumed that KatA promotes phagocyte resistance and thus contributes to SA virulence [137]. However, a *katA*-deficient mutant was not attenuated in a murine-abscess model of SA and all KatA-negative strains retained virulence [138, 139]. This suggested that SA possesses other mechanisms for detoxification of reactive oxygen species (ROS). In fact, two superoxide dismutases were shown to promote SA resistance to ROS *in vitro* however their contribution to SA virulence *in vivo* needs further investigation due to apparently contradictory conclusions [140–142].

Recently it was discovered that staphyloxanthin, the golden pigment of SA, plays a major role in the detoxification of ROS. An isogenic mutant deficient in pigment

formation was constructed by deleting the gene *crtM* encoding dehydrosqualene synthase, a key enzyme in the biosynthetic pathway of staphyloxanthin. Compared to the parental WT, the mutant was more susceptible to oxidative killing, its survival was diminished in the presence of PMNs, and it was less pathogenic in a murine abscess-model [143]. Remarkably, very recently it was discovered that the structure of CrtM resembles that of the mammalian squalene synthetase (SQS) which is involved in the biosynthetic pathways of cholesterol [144]. An SQS inhibitor, which acts as a cholesterol-lowering compound, bound to CrtM with high affinity. When WT SA was grown in the presence of this compound the bacteria lost their ability to produce staphyloxanthin, exhibited increased susceptibility to killing by hydrogen peroxide, were less able to survive in whole human blood and finally were attenuated in a systemic model of infection [144]. This discovery may provide a new strategy for treatment of SA infections by inhibition of staphyloxanthin synthesis [144].

The host has developed additional strategies to cope with the increased resistance of SA to ROS. By using imaging mass spectrometry it was recently found that the PMN protein calprotectin is localized inside abscessed tissue in a murine model of SA abscess formation [145]. Calprotectin belongs to the S100 protein family. It is a complex of two calcium-binding proteins that is abundant in the cytosolic fraction of PMNs and high levels of calprotectin are present in extracellular fluids during various inflammatory conditions [146]. Using the murine model of abscess formation it was shown that calprotectin acts against bacterial infections by chelating Mn^{2+}. Mice with calprotectin deficiency exhibited an increased number of abscesses with higher bacterial loads containing increased amounts of Mn^{2+} compared to WT infected mice [145]. As Mn^{2+} is essential for the activity of SA superoxide dismutase A [141], it is possible that calprotectin not only acts by lowering the concentration of this essential trace element but also by weakening bacterial resistance to ROS. Whether or not calprotectin is specifically secreted during SA abscess formation from live PMNs or released from dead PMNs and acts posthumously, remains to be determined.

The remarkable ability of CA-MRSA to cause severe SA infections in previously healthy individuals as compared to hospital acquired MRSA that mainly affects patients with predisposing risk factors, suggests that the former has acquired an increased pathogenic potential. Since the most prominent CA-MRSA isolates survive relatively well inside PMNs and subsequently cause cell lysis, it was suggested that these strains have evolved an increased ability to resist killing by PMNs and have acquired new means of PMN disruption [147, 148]. The obvious candidate for this lytic activity is the bi-component Panton-Valentine (PVL) leukocidin. This is because the epidemiological linkage between CA-MRSA strains carrying the *pvl* genes and their ability to cause rapidly progressing necrotizing pneumonia has been established [149]. In fact, it was demonstrated that purified PVL causes dermonecrotic lesions in rabbits and lethal necrotic lesions in the lungs of mice [150, 151]. Nonetheless, the actual contribution of PVL to SA virulence remains a matter of debate. Deletion of *pvl* genes did not affect the ability of pandemic CA-MRSA-derived mutants to cause abscesses or bacteremia in the corresponding

murine models [152]. By contrast, overexpression of PVL from a plasmid reduced mice survival in a pneumonia model [150]. Careful re-evaluation of the role of PVL in the same pneumonia model showed that α-hemolysin rather than the PVL toxin was essential for SA pathogenesis [153]. Thus it is still unclear which SA virulence factor mediates PMN lysis. Recently it was discovered that this lysis is mainly caused by small cytolytic peptides known as phenol-soluble modulins (PSMs) [154]. These peptides exhibit pronounced *in vitro* leukocidal, pro-inflammatory and chemotactic activities and are essential for CA-MRSA pathogenesis as determined in murine models of skin, soft-tissue, abscesses and bacteremia infections [154, 155].

4.3.2 Macrophages (Mϕ)

Several studies conducted in recent years have indicated that Mϕ play a key role in the host innate immune response to GAS and SA infections. Nevertheless, Mϕ have received much less attention than PMNs, and hence much less is known about the molecular mechanisms underlying Mϕ interactions with GAS and SA. Obviously, some of the virulence factors that GAS and SA use to prevent opsonophagocytic killing by PMNs are also applicable to Mϕ as these cells use common receptors for phagocytosis of microbes and elicit similar signaling cascades during the uptake process [156].

Early observations revealed that GAS can survive within pharyngeal epithelial cells and macrophage like-cells of patients with tonsillitis [157]. More recently, using biopsies taken from patients with acute soft-tissue infections, it was clearly demonstrated that Mϕ migrate to the site of infection. Moreover, viable GAS were present intracellularly mainly within Mϕ, even in biopsies collected after prolonged antibiotic treatments. This suggested that Mϕ can serve as a reservoir for viable GAS [158]. This notion was confirmed *in vitro* using human monocyte-derived Mϕ, showing that the secreted GAS cysteine protease SpeB plays a role in the ability of GAS to survive within these Mϕ [158].

An additional factor shown to facilitate GAS survival within Mϕ is SalY, a putative lantibiotic peptide encoded by a gene located on the salivaricin-like operon of GAS [159]. The contribution of SalY to virulence was discovered by screening a library of GAS transposon-induced mutants in a zebrafish model that resembles human NF. In this model, the WT strain produced large necrotic lesions with heavy bacterial loads. There was a paucity of inflammatory cells at the intramuscular injection site but an abundance of bacteria were discovered within splenic Mϕ [159]. In contrast, the *salY*-mutant caused a massive recruitment of inflammatory cells, but no mutants were present in splenic Mϕ [159]. Furthermore, a systemic depletion of Mϕ fully restored the virulence of the mutant, strongly suggesting that SalY is essential for GAS survival within Mϕ [159]. This was further verified *in vitro* by using a murine cell-line of Mϕ [159].

Systemic depletion of Mϕ in mice considerably enhanced their sensitivity to a systemic GAS infection [160]. In this infection model it was shown that Mϕ kill intracellular GAS, at least at early points in time after their uptake [160]. The difference between this and the above-described studies may not represent a fundamental discrepancy but rather could result from a time-dependent process of GAS adaptation to the environmental conditions existing within activated Mϕ. In fact, the peroxide response transcriptional regulator (PerR) was shown to contribute to GAS survival within Mϕ in a study comparing survival of WT GAS and a *perR*-deficient mutant [161] Furthermore, the attenuated survival of the *perR* mutant was rescued by inhibition of the Mϕ oxidative burst [161]. In line with these findings, genome-wide analyses of Mϕ gene expression showed that bactericidal activity against GAS is mediated by generation of an oxidative response [162]. Therefore, $p47^{phox}$ was up-regulated in murine Mϕ infected with GAS and bacterial killing was considerably diminished in blood of $p47^{phox-/-}$ mice as opposed to that of WT mice [162].

Although rabbit and murine models of SA skin infections were established in the 1960s [163, 164], 40 years elapsed before it was shown that Mϕ might constitute an important component of the host innate immune response to SA infections [165]. Histopathological examination of soft tissues taken from mice infected subcutaneously with SA revealed an overwhelming infiltrate of Mϕ around the bacteria but no signs of bacteremia. Nevertheless, a significantly increased level of IL-6 was observed in the serum. The increased IL-6 production further implicates Mϕ as being major players in the immune response to cutaneous SA infection [165].

The contribution of Mϕ to protection from SA infection was also demonstrated in murine models of postoperative enteritis and thermal injuries. Systemic depletion of Mϕ considerably decreased the survival rate of mice after intra-intestinal or intravenous injection of MRSA [166, 167]. In a recent *in-vitro* study using human monocyte-derived Mϕ it was shown that SA can persist intracellularly in vacuoles for 3 to 4 days before escaping into the cytoplasm and causing cell lysis [168]. During this time, Mϕ did not reveal any signs of either apoptosis or necrosis and remained functional [168]. However, priming of Mϕ with interferon-γ eradicated intracellular survival of SA. For intracellular survival SA required α-hemolysin, aureolysin, protein A, sortase A, the alternative sigma factor B and the global regulator *agr*, as isogenic mutants deficient in these components were readily killed [168]. Thus the transient survival of SA within Mϕ may provide the pathogen with a vehicle for dissemination of the infection.

4.4 Conclusions

SA and GAS are leading pathogens causing the largest number of human infections, including life-threatening diseases, in previously healthy children and

adults. At the same time they circulate in a larger reservoir of the population without causing symptoms. Such a broad spectrum of pathogenic potentials is caused by vast arrays of virulence factors whose expression is tightly regulated. These factors enable SA and GAS to rapidly respond to the innate defenses exerted by the different cells and tissues which the pathogens encounter during their infectious processes.

We have acquired increased ability to detect the virulence factors of SA and GAS and to identify the components of the innate immunity brought into play during skin and soft tissue infections. Yet our ability to integrate this knowledge into a vectorial process which will enable us to follow and understand the host–pathogen interactions on a continuous basis during infection is still lacking. For example, we do not understand why on the one hand GAS and SA prevent PMNs chemotaxis, but on the other attract PMNs, survive intracellularly and lyse them. Also, increasing evidence shows that Mϕ play a central role in the containment of SA and GAS infections, but the pathogens' virulence factors impairing Mϕ functions still elude discovery. Finally, we need to assess the relative contributions of PMNs and Mϕ and their modes of cooperation in the resolution of SA and GAS soft-tissue infections.

New technologies including the development of human-relevant animal models, advanced gene manipulation techniques in both the animals and pathogens, enhanced performance of genome-wide investigations, and visualization of infectious processes by intravital microscopy will boost our ability to see the picture in its entirety.

Acknowledgments

We thank Dr Nahum Shpigel (The Hebrew University, Koret School of Veterinary Medicine, Rehovot, Israel) for critical reading of this manuscript. The work of our laboratory was supported by grants from: the USA–Israel Binational Science Foundation and the Israeli Science Foundation administered by the Israel Academy of Science and Humanities. Emanuel Hanski is an international research scholar from the Howard Hughes Medical Institute.

References

1 Carapetis, J.R., *et al.* (2005) The global burden of group a streptococcal diseases. *Lancet Infect. Dis.*, **5** (11), 685–694.

2 O'Loughlin, R.E., *et al.* (2007) The epidemiology of invasive group A streptococcal infection and potential vaccine implications: United States, 2000–2004. *Clin. Infect. Dis.*, **45** (7), 853–862.

3 McCaig, L.F., *et al.* (2006) *Staphylococcus aureus*-associated skin and soft tissue infections in ambulatory care. *Emerg. Infect. Dis.*, **12** (11), 1715–1723.

4 Elston, D.M. (2007) Community-acquired methicillin-resistant *Staphylococcus aureus*. *J. Am. Acad. Dermatol.*, **56** (1), 1–16, quiz 17–20.

5 Lowy, F.D. (1998) *Staphylococcus aureus* infections. *N. Engl. J. Med.*, **339** (8), 520–532.

6 Vinh, D.C., and Embil, J.M. (2005) Rapidly progressive soft tissue infections. *Lancet Infect. Dis.*, **5** (8), 501–513.

7 Vandenesch, F., *et al.* (2003) Community-acquired methicillin-resistant *Staphylococcus aureus* carrying Panton–Valentine leukocidin genes: worldwide emergence. *Emerg. Infect. Dis.*, **9** (8), 978–984.

8 Klevens, R.M., *et al.* (2007) Invasive methicillin-resistant *Staphylococcus aureus* infections in the United States. *JAMA*, **298** (15), 1763–1771.

9 Chavakis, T., Preissner, K.T., and Herrmann, M. (2007) The anti-inflammatory activities of *Staphylococcus aureus*. *Trends Immunol.*, **28** (9), 408–418.

10 Fournier, B., and Philpott, D.J. (2005) Recognition of *Staphylococcus aureus* by the innate immune system. *Clin. Microbiol. Rev.*, **18** (3), 521–540.

11 Iwatsuki, K., *et al.* (2006) Staphylococcal cutaneous infections: invasion, evasion and aggression. *J. Dermatol. Sci.*, **42** (3), 203–214.

12 Kwinn, L.A., and Nizet, V. (2007) How group A Streptococcus circumvents host phagocyte defenses. *Future Microbiol.*, 75–84.2

13 Nizet, V. (2007) Understanding how leading bacterial pathogens subvert innate immunity to reveal novel therapeutic targets. *J. Allergy Clin. Immunol.*, **120** (1), 13–22.

14 Rooijakkers, S.H., van Kessel, K.P., and van Strijp, J.A. (2005) Staphylococcal innate immune evasion. *Trends Microbiol.*, **13** (12), 596–601.

15 Voyich, J.M., Musser, J.M., and DeLeo, F.R. (2004) *Streptococcus pyogenes* and human neutrophils: a paradigm for evasion of innate host defense by bacterial pathogens. *Microbes Infect.*, **6** (12), 1117–1123.

16 Beutler, B. (2004) Innate immunity: an overview. *Mol. Immunol.*, **40** (12), 845–859.

17 Ishii, K.J., *et al.* (2008) Host innate immune receptors and beyond: making sense of microbial infections. *Cell Host Microbe*, **3** (6), 352–363.

18 Medzhitov, R. (2007) Recognition of microorganisms and activation of the immune response. *Nature*, **449** (7164), 819–826.

19 Goodarzi, H., Trowbridge, J., and Gallo, R.L. (2007) Innate immunity: a cutaneous perspective. *Clin. Rev. Allergy Immunol.*, **33**(1–2), 15–26.

20 Aderem, A., and Underhill, D.M. (1999) Mechanisms of phagocytosis in macrophages. *Annu. Rev. Immunol.*, **17**, 593–623.

21 Stuart, L.M., and Ezekowitz, R.A. (2005) Phagocytosis: elegant complexity. *Immunity*, **22** (5), 539–550.

22 Gordon, S., and Taylor, P.R. (2005) Monocyte and macrophage heterogeneity. *Nat. Rev. Immunol.*, **5** (12), 953–964.

23 Imhof, B.A., and Aurrand-Lions, M. (2004) Adhesion mechanisms regulating the migration of monocytes. *Nat. Rev. Immunol.*, **4** (6), 432–444.

24 Ley, K., *et al.* (2007) Getting to the site of inflammation: the leukocyte adhesion cascade updated. *Nat. Rev. Immunol.*, **7** (9), 678–689.

25 Rot, A., and von Andrian, U.H. (2004) Chemokines in innate and adaptive host defense: basic chemokinase grammar for immune cells. *Annu. Rev. Immunol.*, 891–928.22

26 Nathan, C. (2006) Neutrophils and immunity: challenges and opportunities. *Nat. Rev. Immunol.*, **6** (3), 173–182.

27 Segal, A.W. (2005) How neutrophils kill microbes. *Annu. Rev. Immunol.*, **23**, 197–223.

28 Gordon, S. (2007) The macrophage: past, present and future. *Eur. J. Immunol.*, **37** (Suppl. 1), S9–S17.

29 Soehnlein, O., *et al.* (2008) Neutrophil secretion products pave the way for inflammatory monocytes. *Blood*, **112** (4), 1461–1471.

30 Gallo, R.L., and Huttner, K.M. (1998) Antimicrobial peptides: an emerging concept in cutaneous biology. *J. Invest. Dermatol.*, **111** (5), 739–743.

31 Niyonsaba, F., and Ogawa, H. (2005) Protective roles of the skin against infection: implication of naturally

occurring human antimicrobial agents beta-defensins, cathelicidin LL-37 and lysozyme. *J. Dermatol. Sci.*, **40** (3), 157–168.

32 Yamasaki, K., and Gallo, R.L. (2008) Antimicrobial peptides in human skin disease. *Eur. J. Dermatol.*, **18** (1), 11–21.

33 Hancock, R.E., and Chapple, D.S. (1999) Peptide antibiotics. *Antimicrob. Agents Chemother.*, **43** (6), 1317–1323.

34 Lehrer, R.I., and Ganz, T. (1999) Antimicrobial peptides in mammalian and insect host defence. *Curr. Opin. Immunol.*, **11** (1), 23–27.

35 Hale, J.D., and Hancock, R.E. (2007) Alternative mechanisms of action of cationic antimicrobial peptides on bacteria. *Expert Rev. Anti. Infect. Ther.*, **5** (6), 951–959.

36 Dorschner, R.A., *et al.* (2001) Cutaneous injury induces the release of cathelicidin anti-microbial peptides active against group A Streptococcus. *J. Invest. Dermatol.*, **117** (1), 91–97.

37 Nizet, V., *et al.* (2001) Innate antimicrobial peptide protects the skin from invasive bacterial infection. *Nature*, **414** (6862), 454–457.

38 Kisich, K.O., *et al.* (2007) The constitutive capacity of human keratinocytes to kill *Staphylococcus aureus* is dependent on beta-defensin 3. *J. Invest. Dermatol.*, **127** (10), 2368–2380.

39 Kisich, K.O., *et al.* (2008) Defective killing of *Staphylococcus aureus* in atopic dermatitis is associated with reduced mobilization of human beta-defensin-3. *J. Allergy Clin. Immunol.*, **122** (1), 62–68.

40 Peschel, A. (2002) How do bacteria resist human antimicrobial peptides? *Trends Microbiol.*, **10** (4), 179–186.

41 Peschel, A., and Sahl, H.G. (2006) The co-evolution of host cationic antimicrobial peptides and microbial resistance. *Nat. Rev. Microbiol.*, **4** (7), 529–536.

42 Collins, L.V., *et al.* (2002) *Staphylococcus aureus* strains lacking D-alanine modifications of teichoic acids are highly susceptible to human neutrophil killing and are virulence attenuated in mice. *J. Infect. Dis.*, **186** (2), 214–219.

43 Kristian, S.A., *et al.* (2005) D-alanylation of teichoic acids promotes group a streptococcus antimicrobial peptide resistance, neutrophil survival, and epithelial cell invasion. *J. Bacteriol.*, **187** (19), 6719–6725.

44 Kristian, S.A., *et al.* (2003) Alanylation of teichoic acids protects *Staphylococcus aureus* against Toll-like receptor 2-dependent host defense in a mouse tissue cage infection model. *J. Infect. Dis.*, **188** (3), 414–423.

45 Peschel, A., *et al.* (2001) *Staphylococcus aureus* resistance to human defensins and evasion of neutrophil killing via the novel virulence factor MprF is based on modification of membrane lipids with L-lysine. *J. Exp. Med.*, **193** (9), 1067–1076.

46 Peschel, A., *et al.* (1999) Inactivation of the dlt operon in *Staphylococcus aureus* confers sensitivity to defensins, protegrins, and other antimicrobial peptides. *J. Biol. Chem.*, **274** (13), 8405–8410.

47 Sieprawska-Lupa, M., *et al.* (2004) Degradation of human antimicrobial peptide LL-37 by *Staphylococcus aureus*-derived proteinases. *Antimicrob. Agents Chemother.*, **48** (12), 4673–4679.

48 Nyberg, P., Rasmussen, M., and Bjorck, L. (2004) alpha2-Macroglobulin-proteinase complexes protect *Streptococcus pyogenes* from killing by the antimicrobial peptide LL-37. *J. Biol. Chem.*, **279** (51), 52820–52823.

49 Frick, I.M., *et al.* (2003) SIC, a secreted protein of *Streptococcus pyogenes* that inactivates antibacterial peptides. *J. Biol. Chem.*, **278** (19), 16561–16566.

50 Jin, T., *et al.* (2004) *Staphylococcus aureus* resists human defensins by production of staphylokinase, a novel bacterial evasion mechanism. *J. Immunol.*, **172** (2), 1169–1176.

51 Clarke, S.R., *et al.* (2007) The *Staphylococcus aureus* surface protein IsdA mediates resistance to innate defenses of human skin. *Cell Host Microbe*, **1** (3), 199–212.

52 Molne, L., Verdrengh, M., and Tarkowski, A. (2000) Role of neutrophil leukocytes in cutaneous infection caused by *Staphylococcus aureus*. *Infect. Immun.*, **68** (11), 6162–6167.

53 Gonzalez-Barca, E., *et al.* (2001) Predisposing factors and outcome of

Staphylococcus aureus bacteremia in neutropenic patients with cancer. *Eur. J. Clin. Microbiol. Infect. Dis.*, **20** (2), 117–119.

54 Vallalta Morales, M., *et al.* (2006) Group A streptococcal bacteremia: outcome and prognostic factors. *Rev. Esp. Quimioter.*, **19** (4), 367–375.

55 Agarwal, D.S. (1967) Subcutaneous staphylococcal infection in mice. II. The inflammatory response to different strains of staphylococci and micrococci. *Br. J. Exp. Pathol.*, **48** (5), 468–482.

56 Hill, M.J. (1968) A staphylococcal aggressin. *J. Med. Microbiol.*, **1** (1), 31–43.

57 Russell, R.J., *et al.* (1976) Effects of staphylococcal products on locomotion and chemotaxis of human blood neutrophils and monocytes. *J. Med. Microbiol.*, **9** (4), 433–439.

58 Cockerill, F.R., 3rd, *et al.* (1998) Molecular, serological, and clinical features of 16 consecutive cases of invasive streptococcal disease. Southeastern Minnesota Streptococcal Working Group. *Clin. Infect. Dis.*, **26** (6), 1448–1458

59 Norrby-Teglund, A., *et al.* (2001) Evidence for superantigen involvement in severe group A streptococcal tissue infections. *J. Infect. Dis.*, **184** (7), 853–860.

60 Taylor, F.B., Jr., *et al.* (1999) Staging of the baboon response to group A streptococci administered intramuscularly: a descriptive study of the clinical symptoms and clinical chemical response patterns. *Clin. Infect. Dis.*, **29** (1), 167–177.

61 Hidalgo-Grass, C., *et al.* (2004) Effect of a bacterial pheromone peptide on host chemokine degradation in group A streptococcal necrotising soft-tissue infections. *Lancet*, **363** (9410), 696–703.

62 Bakleh, M., *et al.* (2005) Correlation of histopathologic findings with clinical outcome in necrotizing fasciitis. *Clin. Infect. Dis.*, **40** (3), 410–414.

63 Akira, S., and Takeda, K. (2004) Toll-like receptor signalling. *Nat. Rev. Immunol.*, **4** (7), 499–511.

64 Hoebe, K., *et al.* (2005) CD36 is a sensor of diacylglycerides. *Nature*, **433** (7025), 523–527.

65 Takeuchi, O., *et al.* (2000) Cutting edge: preferentially the R-stereoisomer of the mycoplasmal lipopeptide macrophage-activating lipopeptide-2 activates immune cells through a toll-like receptor 2- and MyD88-dependent signaling pathway. *J. Immunol.*, **164** (2), 554–557.

66 Miller, L.S., *et al.* (2006) MyD88 mediates neutrophil recruitment initiated by IL-1R but not TLR2 activation in immunity against *Staphylococcus aureus*. *Immunity*, **24** (1), 79–91.

67 Miller, L.S., *et al.* (2007) Inflammasome-mediated production of IL-1beta is required for neutrophil recruitment against *Staphylococcus aureus in vivo*. *J. Immunol.*, **179** (10), 6933–6942.

68 von Bernuth, H., *et al.* (2008) Pyogenic bacterial infections in humans with MyD88 deficiency. *Science*, **321** (5889), 691–696.

69 Loof, T.G., *et al.* (2007) The contribution of dendritic cells to host defenses against *Streptococcus pyogenes*. *J. Infect. Dis.*, **196** (12), 1794–1803.

70 Loof, T.G., Goldmann, O., and Medina, E. (2008) Immune recognition of *Streptococcus pyogenes* by dendritic cells. *Infect. Immun.*, **76** (6), 2785–2792.

71 Gratz, N., *et al.* (2008) Group A streptococcus activates type I interferon production and MyD88-dependent signaling without involvement of TLR2, TLR4, and TLR9. *J. Biol. Chem.*, **283** (29), 19879–19887.

72 Gomez, M.I., *et al.* (2004) *Staphylococcus aureus* protein A induces airway epithelial inflammatory responses by activating TNFR1. *Nat. Med.*, **10** (8), 842–848.

73 Gomez, M.I., *et al.* (2006) *Staphylococcus aureus* protein A activates TNFR1 signaling through conserved IgG binding domains. *J. Biol. Chem.*, **281** (29), 20190–20196.

74 Gomez, M.I., Seaghdha, M.O., and Prince, A.S. (2007) *Staphylococcus aureus* protein A activates TACE through EGFR-dependent signaling. *EMBO J.*, **26** (3), 701–709.

75 Herwald, H., *et al.* (2004) M protein, a classical bacterial virulence determinant, forms complexes with fibrinogen that induce vascular leakage. *Cell*, **116** (3), 367–379.

76 Pahlman, L.I., *et al.* (2006) Streptococcal M protein: a multipotent and powerful inducer of inflammation. *J. Immunol.*, **177** (2), 1221–1228.

77 Jarva, H., *et al.* (2003) Complement resistance mechanisms of streptococci. *Mol. Immunol.*, **40** (2–4), 95–107.

78 Kraiczy, P., and Wurzner, R. (2006) Complement escape of human pathogenic bacteria by acquisition of complement regulators. *Mol. Immunol.*, **43** (1–2), 31–44.

79 Lindahl, G., Sjobring, U., and Johnsson, E. (2000) Human complement regulators: a major target for pathogenic microorganisms. *Curr. Opin. Immunol.*, **12** (1), 44–51.

80 Rooijakkers, S.H., and van Strijp, J.A. (2007) Bacterial complement evasion. *Mol. Immunol.*, **44** (1–3), 23–32.

81 Springer, T.A. (1995) Traffic signals on endothelium for lymphocyte recirculation and leukocyte emigration. *Annu. Rev. Physiol.*, **57**, 827–872.

82 Le, Y., Murphy, P.M., and Wang, J.M. (2002) Formyl-peptide receptors revisited. *Trends Immunol.*, **23** (11), 541–548.

83 Guo, R.F., and Ward, P.A. (2005) Role of C5a in inflammatory responses. *Annu. Rev. Immunol.*, **23**, 821–852.

84 Chen, C.C., and Cleary, P.P. (1990) Complete nucleotide sequence of the streptococcal C5a peptidase gene of *Streptococcus pyogenes*. *J. Biol. Chem.*, **265** (6), 3161–3167.

85 Siezen, R.J., *et al.* (1991) Homology modelling and protein engineering strategy of subtilases, the family of subtilisin-like serine proteinases. *Protein Eng.*, **4** (7), 719–737.

86 Cleary, P.P., *et al.* (1992) Streptococcal C5a peptidase is a highly specific endopeptidase. *Infect. Immun.*, **60** (12), 5219–5223.

87 Kawai, M., *et al.* (1991) Identification and synthesis of a receptor binding site of human anaphylatoxin C5a. *J. Med. Chem.*, **34** (7), 2068–2071.

88 Anderson, E.T., *et al.* (2002) Processing, stability, and kinetic parameters of C5a peptidase from *Streptococcus pyogenes*. *Eur. J. Biochem.*, **269** (19), 4839–4851.

89 Berge, A., and Bjorck, L. (1995) Streptococcal cysteine proteinase releases biologically active fragments of streptococcal surface proteins. *J. Biol. Chem.*, **270** (17), 9862–9867.

90 Terao, Y., *et al.* (2006) Multifunctional glyceraldehyde-3-phosphate dehydrogenase of *Streptococcus pyogenes* is essential for evasion from neutrophils. *J. Biol. Chem.*, **281** (20), 14215–14223.

91 Ji, Y., *et al.* (1996) C5a peptidase alters clearance and trafficking of group A streptococci by infected mice. *Infect. Immun.*, **64** (2), 503–510.

92 Hidalgo-Grass, C., *et al.* (2006) A streptococcal protease that degrades CXC chemokines and impairs bacterial clearance from infected tissues. *EMBO J.*, **25** (19), 4628–4637.

93 Bohnsack, J.F., Chang, J.K., and Hill, H.R. (1993) Restricted ability of group B streptococcal C5a-ase to inactivate C5a prepared from different animal species. *Infect. Immun.*, **61** (4), 1421–1426.

94 Edwards, R.J., *et al.* (2005) Specific C-terminal cleavage and inactivation of interleukin-8 by invasive disease isolates of *Streptococcus pyogenes*. *J. Infect. Dis.*, **192** (5), 783–790.

95 Sumby, P., *et al.* (2008) A chemokine-degrading extracellular protease made by group A Streptococcus alters pathogenesis by enhancing evasion of the innate immune response. *Infect. Immun.*, **76** (3), 978–985.

96 Zinkernagel, A.S., *et al.* (2008) The IL-8 protease SpyCEP/ScpC of group A Streptococcus promotes resistance to neutrophil killing. *Cell Host Microbe*, **4** (2), 170–178.

97 Gupta, A.K., *et al.* (2005) Induction of neutrophil extracellular DNA lattices by placental microparticles and IL-8 and their presence in preeclampsia. *Hum. Immunol.*, **66** (11), 1146–1154.

98 Hussain, M., *et al.* (2002) Insertional inactivation of Eap in *Staphylococcus aureus* strain Newman confers reduced

staphylococcal binding to fibroblasts. *Infect. Immun.*, **70** (6), 2933–2940.

99 Palma, M., Haggar, A., and Flock, J.I. (1999) Adherence of *Staphylococcus aureus* is enhanced by an endogenous secreted protein with broad binding activity. *J. Bacteriol.*, **181** (9), 2840–2845.

100 Chavakis, T., *et al.* (2002) *Staphylococcus aureus* extracellular adherence protein serves as anti-inflammatory factor by inhibiting the recruitment of host leukocytes. *Nat. Med.*, **8** (7), 687–693.

101 Athanasopoulos, A.N., *et al.* (2006) The extracellular adherence protein (Eap) of *Staphylococcus aureus* inhibits wound healing by interfering with host defense and repair mechanisms. *Blood*, **107** (7), 2720–2727.

102 de Haas, C.J., *et al.* (2004) Chemotaxis inhibitory protein of *Staphylococcus aureus*, a bacterial antiinflammatory agent. *J. Exp. Med.*, **199** (5), 687–695.

103 Postma, B., *et al.* (2004) Chemotaxis inhibitory protein of *Staphylococcus aureus* binds specifically to the C5a and formylated peptide receptor. *J. Immunol.*, **172** (11), 6994–7001.

104 Haas, P.J., *et al.* (2005) The structure of the C5a receptor-blocking domain of chemotaxis inhibitory protein of *Staphylococcus aureus* is related to a group of immune evasive molecules. *J Mol Biol*, **353** (4), 859–872.

105 Wright, A.J., *et al.* (2007) Characterisation of receptor binding by the chemotaxis inhibitory protein of *Staphylococcus aureus* and the effects of the host immune response. *Mol. Immunol.*, **44** (10), 2507–2517.

106 Todd, E., and Lancefield, R. (1928) Variants of hemolytic streptococci; their relation to type-specific substance, virulence, and toxin. *J. Exp. Med.*, **48**, 751–767.

107 Ward, H., and Lyons, C. (1935) Studies on the hemolytic streptococcus of human origin. I. Observations on the virulent, attenuated, and avirulent variants. *J. Exp. Med.*, **61**, 515–529.

108 Ashbaugh, C.D., *et al.* (1998) Molecular analysis of the role of the group A streptococcal cysteine protease, hyaluronic acid capsule, and M protein in a murine model of human invasive soft-tissue infection. *J. Clin. Invest.*, **102** (3), 550–560.

109 Jacks-Weis, J., Kim, Y., and Cleary, P.P. (1982) Restricted deposition of C3 on M+ group A streptococci: correlation with resistance to phagocytosis. *J. Immunol.*, **128** (4), 1897–1902.

110 Lancefield, R.C. (1962) Current knowledge of type-specific M antigens of group A streptococci. *J. Immunol.*, **89**, 307–313.

111 Sutterwala, F.S., Rosenthal, L.A., and Mosser, D.M. (1996) Cooperation between CR1 (CD35) and CR3 (CD 11b/CD18) in the binding of complement-opsonized particles. *J. Leukoc. Biol.*, **59** (6), 883–890.

112 Ehlers, M.R. (2000) CR3: a general purpose adhesion-recognition receptor essential for innate immunity. *Microbes Infect.*, **2** (3), 289–294.

113 DeMaster, E., *et al.* (2002) M(+) group a streptococci are phagocytized and killed in whole blood by C5a-activated polymorphonuclear leukocytes. *Infect. Immun.*, **70** (1), 350–359.

114 Staali, L., *et al.* (2003) *Streptococcus pyogenes* expressing M and M-like surface proteins are phagocytosed but survive inside human neutrophils. *Cell Microbiol.*, **5** (4), 253–265.

115 Staali, L., *et al.* (2006) *Streptococcus pyogenes* bacteria modulate membrane traffic in human neutrophils and selectively inhibit azurophilic granule fusion with phagosomes. *Cell Microbiol.*, **8** (4), 690–703.

116 Voyich, J.M., *et al.* (2003) Genome-wide protective response used by group A Streptococcus to evade destruction by human polymorphonuclear leukocytes. *Proc. Natl Acad. Sci. USA*, **100** (4), 1996–2001.

117 Voyich, J.M., *et al.* (2004) Engagement of the pathogen survival response used by group A Streptococcus to avert destruction by innate host defense. *J. Immunol.*, **173** (2), 1194–1201.

118 King, K.Y., Horenstein, J.A., and Caparon, M.G. (2000) Aerotolerance and peroxide resistance in peroxidase and PerR mutants of *Streptococcus pyogenes*. *J. Bacteriol.*, **182** (19), 5290–5299.

119 Brenot, A., *et al.* (2004) Contribution of glutathione peroxidase to the virulence of *Streptococcus pyogenes*. *Infect. Immun.*, **72** (1), 408–413.

120 Nathan, C. (2002) Points of control in inflammation. *Nature*, **420** (6917), 846–852.

121 Kobayashi, S.D., *et al.* (2003) Bacterial pathogens modulate an apoptosis differentiation program in human neutrophils. *Proc. Natl Acad. Sci. USA*, **100** (19), 10948–10953.

122 Brinkmann, V., *et al.* (2004) Neutrophil extracellular traps kill bacteria. *Science*, **303** (5663), 1532–1535.

123 Fuchs, T.A., *et al.* (2007) Novel cell death program leads to neutrophil extracellular traps. *J. Cell Biol.*, **176** (2), 231–241.

124 Urban, C.F., *et al.* (2006) Neutrophil extracellular traps capture and kill *Candida albicans* yeast and hyphal forms. *Cell Microbiol.*, **8** (4), 668–676.

125 Sumby, P., *et al.* (2005) Extracellular deoxyribonuclease made by group A Streptococcus assists pathogenesis by enhancing evasion of the innate immune response. *Proc. Natl Acad. Sci. USA*, **102** (5), 1679–1684.

126 Buchanan, J.T., *et al.* (2006) DNase expression allows the pathogen group A Streptococcus to escape killing in neutrophil extracellular traps. *Curr. Biol.*, **16** (4), 396–400.

127 Jongerius, I., *et al.* (2007) Staphylococcal complement evasion by various convertase-blocking molecules. *J. Exp. Med.*, **204** (10), 2461–2471.

128 Rooijakkers, S.H., *et al.* (2005) Immune evasion by a staphylococcal complement inhibitor that acts on C3 convertases. *Nat. Immunol.*, **6** (9), 920–927.

129 Rooijakkers, S.H., *et al.* (2006) Early expression of SCIN and CHIPS drives instant immune evasion by *Staphylococcus aureus*. *Cell Microbiol.*, **8** (8), 1282–1293.

130 Van de Velde, H. (1895) Etude sur le mechanisme de la virulence du staphylocoque pyogene. *Cellule*, **10**, 51–54.

131 Kapral, F.A., and Shayegani, M.G. (1959) Intracellular survival of staphylococci. *J. Exp. Med.*, **110** (1), 123–138.

132 Melly, M.A., Thomison, J.B., and Rogers, D.E. (1960) Fate of staphylococci within human leukocytes. *J. Exp. Med.*, **112**, 1121–1130.

133 Rogers, D.E., and Melly, M.A. (1960) Further observations on the behavior of staphylococci within human leukocytes. *J. Exp. Med.*, **111**, 533–558.

134 Gresham, H.D., *et al.* (2000) Survival of *Staphylococcus aureus* inside neutrophils contributes to infection. *J. Immunol.*, **164** (7), 3713–3722.

135 Medina, E., *et al.* (2003) Survival of *Streptococcus pyogenes* within host phagocytic cells: a pathogenic mechanism for persistence and systemic invasion. *J. Infect. Dis.*, **187** (4), 597–603.

136 Medina, E., Rohde, M., and Chhatwal, G.S. (2003) Intracellular survival of *Streptococcus pyogenes* in polymorphonuclear cells results in increased bacterial virulence. *Infect. Immun.*, **71** (9), 5376–5380.

137 Mandell, G.L. (1975) Catalase, superoxide dismutase, and virulence of *Staphylococcus aureus*. *In vitro* and *in vivo* studies with emphasis on staphylococcal–leukocyte interaction. *J. Clin. Invest.*, **55** (3), 561–566.

138 Horsburgh, M.J., Ingham, E., and Foster, S.J. (2001) In *Staphylococcus aureus*, fur is an interactive regulator with PerR, contributes to virulence, and is necessary for oxidative stress resistance through positive regulation of catalase and iron homeostasis. *J. Bacteriol.*, **183** (2), 468–475.

139 Messina, C.G., *et al.* (2002) Catalase negative *Staphylococcus aureus* retain virulence in mouse model of chronic granulomatous disease. *FEBS Lett.*, **518** (1–3), 107–110.

140 Clements, M.O., Watson, S.P., and Foster, S.J. (1999) Characterization of the major superoxide dismutase of *Staphylococcus aureus* and its role in starvation survival, stress resistance, and pathogenicity. *J. Bacteriol.*, **181** (13), 3898–3903.

141 Karavolos, M.H., *et al.* (2003) Role and regulation of the superoxide dismutases of *Staphylococcus aureus*. *Microbiology*, **149** (Pt 10), 2749–2758.

142 Schneider, W.P., *et al.* (2002) Virulence gene identification by differential fluorescence induction analysis of *Staphylococcus aureus* gene expression during infection-simulating culture. *Infect. Immun.*, **70** (3), 1326–1333.

143 Liu, G.Y., *et al.* (2005) *Staphylococcus aureus* golden pigment impairs neutrophil killing and promotes virulence through its antioxidant activity. *J. Exp. Med.*, **202** (2), 209–215.

144 Liu, C.I., *et al.* (2008) A cholesterol biosynthesis inhibitor blocks *Staphylococcus aureus* virulence. *Science*, **319** (5868), 1391–1394.

145 Corbin, B.D., *et al.* (2008) Metal chelation and inhibition of bacterial growth in tissue abscesses. *Science*, **319** (5865), 962–965.

146 Yui, S., Nakatani, Y., and Mikami, M. (2003) Calprotectin (S100A8/S100A9), an inflammatory protein complex from neutrophils with a broad apoptosis-inducing activity. *Biol. Pharm. Bull.*, **26** (6), 753–760.

147 Li, M., *et al.* (2007) The antimicrobial peptide-sensing system aps of *Staphylococcus aureus*. *Mol. Microbiol.*, **66** (5), 1136–1147.

148 Palazzolo-Ballance, A.M., *et al.* (2008) Neutrophil microbicides induce a pathogen survival response in community-associated methicillin-resistant *Staphylococcus aureus*. *J. Immunol.*, **180** (1), 500–509.

149 Gillet, Y., *et al.* (2002) Association between *Staphylococcus aureus* strains carrying gene for Panton–Valentine leukocidin and highly lethal necrotising pneumonia in young immunocompetent patients. *Lancet*, **359** (9308), 753–759.

150 Labandeira-Rey, M., *et al.* (2007) *Staphylococcus aureus* Panton–Valentine leukocidin causes necrotizing pneumonia. *Science*, **315** (5815), 1130–1133.

151 Ward, P.D., and Turner, W.H. (1980) Identification of staphylococcal Panton–Valentine leukocidin as a potent dermonecrotic toxin. *Infect. Immun.*, **28** (2), 393–397.

152 Voyich, J.M., *et al.* (2006) Is Panton–Valentine leukocidin the major virulence determinant in community-associated methicillin-resistant *Staphylococcus aureus* disease? *J. Infect. Dis.*, **194** (12), 1761–1770.

153 Bubeck Wardenburg, J., *et al.* (2007) Poring over pores: alpha-hemolysin and Panton–Valentine leukocidin in *Staphylococcus aureus* pneumonia. *Nat. Med.*, **13** (12), 1405–1406.

154 Wang, R., *et al.* (2007) Identification of novel cytolytic peptides as key virulence determinants for community-associated MRSA. *Nat. Med.*, **13** (12), 1510–1514.

155 Diep, B.A., and Otto, M. (2008) The role of virulence determinants in community-associated MRSA pathogenesis. *Trends Microbiol.*, **16** (8), 361–369.

156 Underhill, D.M., and Ozinsky, A. (2002) Phagocytosis of microbes: complexity in action. *Annu. Rev. Immunol.*, **20**, 825–852.

157 Osterlund, A., and Engstrand, L. (1995) Intracellular penetration and survival of *Streptococcus pyogenes* in respiratory epithelial cells *in vitro*. *Acta Otolaryngol.*, **115** (5), 685–688.

158 Thulin, P., *et al.* (2006) Viable group A streptococci in macrophages during acute soft tissue infection. *PLoS Med.*, **3** (3), e53.

159 Phelps, H.A., and Neely, M.N. (2007) SalY of the *Streptococcus pyogenes* lantibiotic locus is required for full virulence and intracellular survival in macrophages. *Infect. Immun.*, **75** (9), 4541–4551.

160 Goldmann, O., *et al.* (2004) Role of macrophages in host resistance to group A streptococci. *Infect. Immun.*, **72** (5), 2956–2963.

161 Gryllos, I., *et al.* (2008) PerR confers phagocytic killing resistance and allows pharyngeal colonization by group A streptococcus. *PLoS Pathog.*, **4** (9), e1000145.

162 Goldmann, O., *et al.* (2007) Transcriptome analysis of murine macrophages in response to infection with *Streptococcus pyogenes* reveals an unusual activation program. *Infect. Immun.*, **75** (8), 4148–4157.

163 Goshi, K., *et al.* (1961) Studies on the pathogenesis of staphylococcal infection. II. The effect of non-specific

inflammation. *J. Exp. Med.*, 249–257.113

164 Noble, W.C. (1965) The production of subcutaneous staphylococcal skin lesions in mice. *Br. J. Exp. Pathol.*, **46** (3), 254–262.

165 Molne, L., and Tarkowski, A. (2000) An experimental model of cutaneous infection induced by superantigen-producing *Staphylococcus aureus*. *J. Invest. Dermatol.*, **114** (6), 1120–1125.

166 Katakura, T., *et al.* (2005) Immunological control of methicillin-resistant *Staphylococcus aureus* (MRSA) infection in an immunodeficient murine model of thermal injuries. *Clin. Exp. Immunol.*, **142** (3), 419–425.

167 Tanaka, H., *et al.* (2004) Role of macrophages in a mouse model of postoperative MRSA enteritis. *J. Surg. Res.*, **118** (2), 114–121.

168 Kubica, M., *et al.* (2008) A potential new pathway for *Staphylococcus aureus* dissemination: the silent survival of *S. aureus* phagocytosed by human monocyte-derived macrophages. *PLoS ONE*, **3** (1), e1409.

5
Mechanisms of Meningeal Invasion by Septicemic Extracellular Pathogens: The Examples of *Neisseria meningitidis*, *Streptococcus agalactiae* and *Escherichia coli*

Olivier Join-Lambert, Etienne Carbonnelle, Fabrice Chrétien, Sandrine Bourdoulous, Stéphane Bonacorsi, Claire Poyart, and Xavier Nassif

5.1
Introduction

Bacterial meningitis is a leading cause of central nervous system infections. It is an inflammatory process that results from bacterial entry into the subarachnoidal spaces (SAS). Bacterial meningitis can be due to the dissemination of contiguous infections such as sinusitis or mastoiditis of the meningeal membranes, but most cases are caused by blood-borne pathogens. Unlike other peripheral organs, the central nervous system (CNS) is protected from blood-borne insults by specialized structures that separate the nervous parenchyma and the cerebrospinal fluid from the blood. These blood–brain barriers (BBB) effectively protect the CNS from most circulating bacteria, restricting the etiology of bacterial meningitis to a few and predominantly extracellular pathogens: *Escherichia coli* K1 and *Streptococcus agalactiae* (Group B Streptococcus) in the newborn, *Neisseria meningitidis*, *Haemophilus influenzae* type b and *Streptococcus pneumoniae* in children and adults [1–3]. It has been estimated that 63 and 9% of bacteremia due to *N. meningitidis* and *S. pneumoniae,* respectively, are associated with meningitis [4], demonstrating that the ability of these bacteria to enter the subarachnoidal spaces varies. Paradoxically, these bacteria are commensal in the nasopharynx (*N. meningtidis*, *S. pneumoniae* and *H. influenzae*) and digestive tract (*E. coli*, *S. agalactiae*) [5].

The pathophysiology of bacterial meningitis is thus a multi-step process that reflects the ability of bacterial pathogens to cross the oropharyngeal or digestive mucosal barrier, to survive and to multiply in bloodstream and finally, to cross the blood–brain barrier [5–7]. As stated above, the number of bacteria capable of invading the meninges is limited, suggesting that specific virulence factors are required for bacteria to enter the subarachnoidal space. Indeed it has been demonstrated that these extracellular pathogens express various virulence factors which allow them to survive in the extracellular fluids and eventually to interact directly with the components of the blood–brain barrier. The main virulence factor expressed

Bacterial Virulence: Basic Principles, Models and Global Approaches. Edited by Philippe Sansonetti
© 2010 WILEY-VCH Verlag GmbH & Co. KGaA, Weinheim
ISBN: 978-3-527-32326-5

by all extracellular pathogens is a capsule that prevents bacterial phagocytosis or complement-mediated lysis [8–13]. Once inside the CSF, bacterial multiplication is thought to be uncontrolled, due to the local deficiency in complement and immunoglobulins and despite the influx of polymorphonuclear leukocytes induced by the local inflammatory response. However data obtained in primates showed that bacterial presence in the CSF can be transient if bacteremia is not sustained, reflecting the fact that bacterial entry into the CSF may not always lead to meningitis [14]. Compared to intracellular pathogens such as *Listeria monocytogenes* that are responsible for both meningitis and encephalitis [15, 16], extracellular bacterial pathogens are only responsible for meningitis, thus suggesting that these bacteria are unable to 'successfully' cross the parenchymal blood–brain barrier, and/or to induce an inflammation, and/or multiply in the brain parenchyma. Severe medical complications of bacterial meningitis such as cerebral edema, and vasculitis occur in 20 to 30% of treated patients and are due to the local inflammatory response and not to bacterial invasion of the brain parenchyma [17, 18].

In the last 30 years, most research carried on bacterial meningitis has concentrated on the understanding of the interactions of pathogens with the BBB using *in vitro* models. However, the mechanism(s) and site(s) by which bacteria cross the blood–CSF barrier *in vivo* remain undefined. In this chapter, we will first describe the different structures that form the barriers between the blood and the brain, and discuss the different routes that may be used by extracellular bacteria to invade the subarachnoid space. We will finally address specific aspects of the meningeal invasion of major extracellular pathogens, focusing on those bacteria that need to be blood-borne in order to invade the meninges, i.e. *E. coli*, *S. agalactiae* and *N. meningitidis*.

5.2
Bacterial Translocation through the Central Nervous System Vasculature

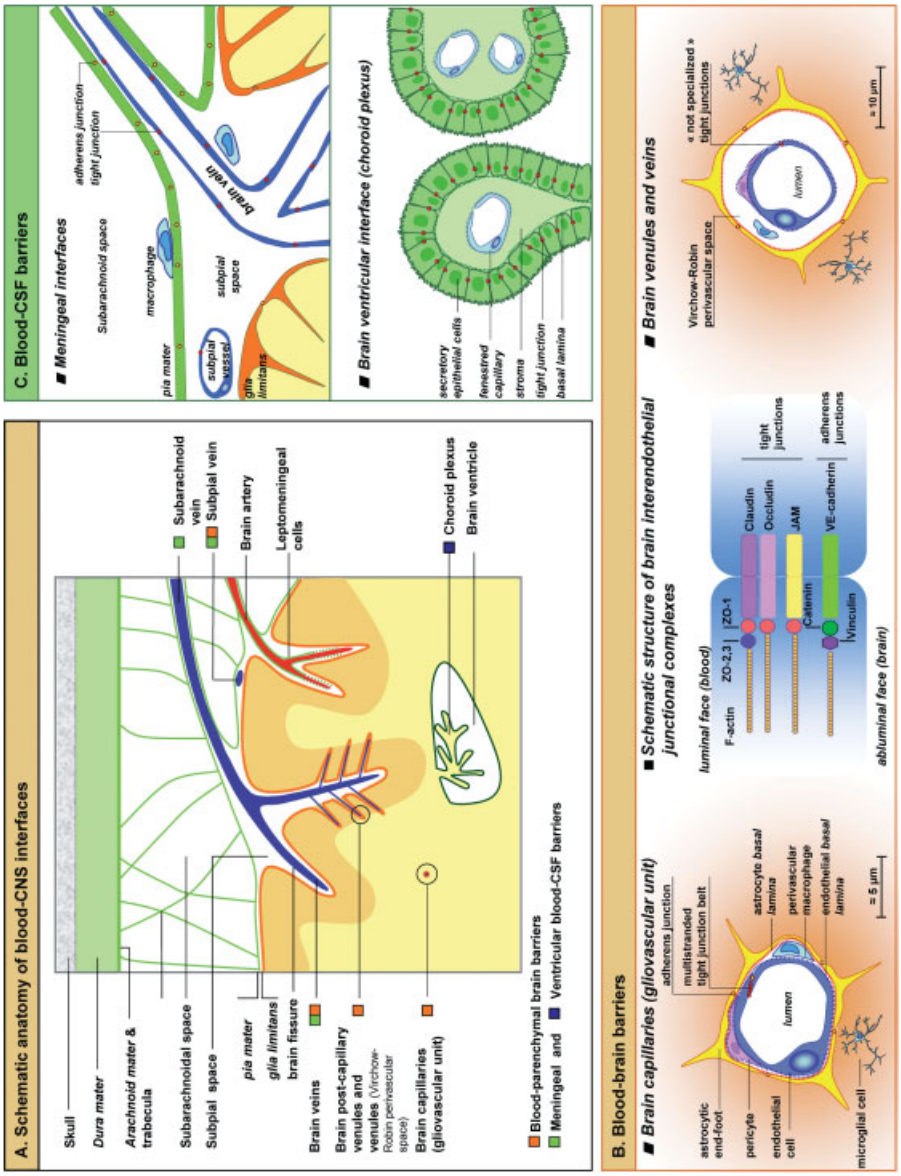
5.2.1
Structural and Functional Heterogeneity of the Blood–CNS Interfaces

History of the 'Blood–Brain Barrier' The concept of the 'blood-brain barrier' results from the original observations of Ehrlich in the late nineteenth century, and from later observations by Goldman and Lewandowsky who reported that the brain remained as 'white as snow' after intravenous injection of intravital dyes such as trypan blue [19, 20]. From these experiments, it was proposed that brain vessels form a specialized interface that controls the exchanges of solutes and cells between the blood and the brain parenchyma. The concept of the 'blood–brain barrier' corresponds to the different blood–CNS interfaces that have been described Figure 5.1A. Anatomical, structural and functional particularities of the brain vasculature and perivascular cells are of critical importance to assess the potential sites and mechanisms of entry of bacterial pathogens into the subarachnoidal space.

Anatomy of the Blood–Brain Barrier Schematically, the blood flow of the CNS is predominantly supplied by the internal carotid arteries (70% of the cerebral blood flow) and by the vertebral arteries. In the skull base, before entering into the CNS, large arteries cross the meningeal spaces where they anastomose to form the circle of Willis [21]. Meninges are composed of three connective tissue layers that surround the brain and spinal cord (Figure 5.1A). The outermost membrane, the dura mater or pachymeninges, is a tough and rigid fibrous connective tissue that is apposed to the internal side of the skull. The two other layers are called leptomeninges. The innermost membrane is the pia mater which is composed of a thin monolayer of meningeal (suppressed) cells and few collagen fibers that cover the CNS. The arachnoid lies between the dura and pia mater. It is composed of an external outer membrane lining the dura mater and arachnoidal trabeculae that form sheet or fine filiform structures that cross the subarachnoidal space to join the pia mater. The pia mater and the outer arachnoidal membrane delineate the subarachnoidal space (SAS) into which the CSF flows. All vessels that circulate within the SAS are located within arachnoidal trabeculae, and are therefore separated from the CSF by a layer of leptomeningeal cells. Branches of CNS arteries penetrate into the nervous parenchyma within their leptomeningeal cell sheathe which progressively disappears [22]. When CNS veins exit the parenchyma, they enter the subpial space either directly or through small fissures where they are neither ensheathed by glial or leptomeningeal cells (Figure 5.1A). Then they cross the subarachnoidal space to turn toward the dural sinuses of the dura mater, a unique structure of the venous drainage system of the brain. It has now been established that the subpial space does not communicate directly with the subarachnoidal space but that the pia mater which covers the surface of the CNS is deflected around the veins that enter the subarachnoidal space to form the outer arachnoidal membrane which coats the meningeal veins (Figure 5.1A). Thus, the subpial space is both confluent with the parenchymal perivascular venous Virchow–Robin spaces, and with the perivascular space of the subarachnoidal veins [23].

5.2.1.1 The Blood–Brain Barrier Sensu Stricto

The role of the blood–brain barrier is to maintain nervous tissue homeostasis and to protect the CNS from circulating blood-borne insults. CNS capillaries represent the most important vascular surface contact between the nervous tissue and the blood. At this anatomical level, the BBB is formed by specialized endothelial cells via a peculiar organization of the basal lamina and perivascular cells that form a second line of defense and actively control the permeability of the endothelial monolayer. CNS capillary endothelial cells have at least two specific major features: (i) the presence of specialized junctional complexes; and (ii) a sparse pinocytotic vesicular transport activity that is counterbalanced by highly specialized transport systems that limit the entry of neuroactive blood-borne molecules [24]. Junctional complexes are composed of adherent junctions and of multi-stranded belts of tight or occludens junctions that result in apparent membrane fusion of adjacent endothelial cells, forming a continuous blood vessel (Figure 5.1B). Tight


A. Schematic anatomy of blood-CNS interfaces
Skull
Dura mater
Arachnoid mater & trabecula
Subarachnoidal space
Subpial space
pia mater
glia limitans
brain fissure
Brain veins
Brain post-capillary venules and venules (Virchow-Robin perivascular space)
Brain capillaries (gliovascular unit)
Subarachnoid vein
Subpial vein
Brain artery
Leptomeningeal cells
Choroid plexus
Brain ventricle
Blood-parenchymal brain barriers
Meningeal and
Ventricular blood-CSF barriers
C. Blood-CSF barriers
Meningeal interfaces
adherens junction
tight junction
Subarachnoid space
macrophage
pia mater
subpial space
Dorlin Vohn
subpial vessel
glia limitans
Brain ventricular interface (choroid plexus)
secretory epithelial cells
fenestred capillary
stroma
tight junction
basal lamina
B. Blood-brain barriers
Brain capillaries (gliovascular unit)
adherens junction
multistranded tight junction belt
astrocytic end-foot
pericyte
endothelial cell
astrocyte basal lamina
perivascular macrophage
endothelial basal lamina
microglial cell
lumen
≈ 5 µm
Schematic structure of brain interendothelial junctional complexes
luminal face (blood)
F-actin
ZO-1
ZO-2,3
Claudin
Occludin
tight junctions
JAM
Catenin
VE-cadherin
adherens junctions
Vinculin
abluminal face (brain)
Brain venules and veins
Virchow-Robin perivascular space
« not specialized » tight junctions
lumen
≈ 10 µm

interendothelial junctions exclude the paracellular passage of hydrophilic macromolecules or particles between the blood and the CNS and accounts for the high endothelial electric resistance of CNS capillaries [25–27]. *In vitro*, the specialization of tight junctions of CNS capillaries has been shown to be under the control of paracrine factors such as angiotensin II which is produced by astrocytic cellular extensions (astrocytic end-feet) that unsheathe CNS capillaries (Figure 5.1B) [28, 29]. At this level, the basal lamina of both the endothelial cells and astrocytic processes which are produced by astrocytes and endothelial cells, are in very close contact and may be fused. For this reason, the blood–brain barrier at the capillary level is also known as the 'gliovascular' or 'neurovascular' unit. Astrocytes form the primitive blood–brain barrier in non-mammalian organisms [30] and form a second line of defense that separates the nervous parenchyma from the perivascular space [31]. At the periphery of the cortex (Figure 5.1A), astrocyte processes form the so-called glia limitans. They are involved in the immune surveillance of the perivascular spaces and use a variety of methods to fulfill this function: they restrict the entry of T lymphocytes into the nervous tissue by inducing their apoptosis [32] and they can be activated via numerous toll-like receptors leading to the

Figure 5.1 Anatomy and heterogeneity of the blood–brain barriers. (A) Blood CNS interfaces. The blood–brain barrier, *stricto sensu*, is localized within the brain parenchyma. It includes brain capillaries, brain veins and venules. The blood–CSF barrier comprises two distinct localizations: the ventricular (choroid plexus) and the meningeal (subpial veins, subarachnoid veins) blood–CSF interfaces. For a precise description of the meningeal structures, see in text. (B) The blood–brain barrier. The endothelial cells of brain capillaries form a continuous barrier to blood components due to the presence of intercellular multi-stranded tight junction complexes that are continuously deployed around each cell, preventing the paracellular diffusion of non-lipophilic blood components. Interendothelial junctional complexes also comprise adherens junction at the basolateral endothelial cell membrane. Tight junctions are formed by transmembrane proteins such as claudin, occludin and the junctional adhesion molecule JAM, whereas VE-cadherin is the main component of adherens junctions. Their extracellular domains interact in a homophilic manner, whereas their intracellular domains are anchored to cytoplasmic proteins such as zonula occludens 1, 2 and 3, catenins and vinculin which in turn interact with the cellular actin cytosquelon (F-actin). Perivascular astrocytic end-feet ensheathe capillary endothelial cells and control the permeability of the endothelial barrier. Other perivascular cells include pericytes, perivascular macrophages and microglial cells. Contrary to observations at the capillary level, astrocytes are not in close contact with the endothelial cells of brain postcapillary venules and veins and the structure of interendothelial tight junctions is more leaky that than in the parenchyma. (C) The blood–CSF barrier. Subarachnoid veins form the meningeal blood–CSF barrier. When brain veins enter the subarachnoid space, they are ensheathed by leptomeningeal cells that are linked by adherens junctions. At the surface of the brain, arachnoid trabeculae fuse with the pia mater. The subpial space is therefore not contiguous with the subarachnoidal space. In contrast to brain arteries that enter the brain via the leptomeninges, brain veins circulate free within the subpial space and brain fissures. Since blood capillaries of the choroids plexus are fenestrated, the ventricular blood–CSF barrier is provided by epithelial secretory cells that delineate the choroids plexus.

production of pro-inflammatory mediators in response to diverse stimuli, including bacterial ligands [31, 33]. Other perivascular cells including pericytes, perivascular CNS-resident macrophages and microglial cells play an important role in the immune surveillance of the CNS and probably determine the fate of bacteria that may have crossed the endothelial interface [34–37]. At the level of CNS capillaries, the blood–brain barrier is thus a highly specialized structure that is particularly efficient at preventing the entry of extracellular bacteria into the brain parenchyma.

Anatomically the intracerebral venous network is part of the blood–brain barrier (Figure 5.1A). Nevertheless, due to the progressive emergence of the Virchow–Robin perivascular spaces, CNS post-capillary veins and venules are not as close to the CNS parenchyma as CNS capillaries (Figure 5.1B). An important point is that the structure of CNS veins differs strikingly from that of CNS capillaries. In contrast to CNS capillaries, the venous blood–brain barrier does not completely prevent the exit of blood tracers into the perivascular space [38–40]. It is not known whether a paracellular or transcellular diffusion mechanism accounts for this observation. Electron microscopy studies have shown that the endothelial cells of CNS post-capillary venules are also interconnected by tight junctions, but that their organization is not as tight as that of CNS capillaries. A possible explanation is that astrocytic end-feet require intimate interactions with endothelial cells to induce a 'full' BBB phenotype [29, 31]. As stated above, when capillaries become venules, endothelial cells of the CNS draining system are progressively separated from astrocytic end-feet by the Virchow–Robin perivascular spaces. The distance between endothelial cells and astrocyte processes is even more important when veins exit the CNS parenchyma in the subpial space (Figure 5.1A,C). These findings highlight the fact that the venous system of the CNS can be considered to be the Achilles' heel of the blood–brain barrier and that it is not the presence of tight junctions but their organization that is specific to the capillary blood–brain barrier.

5.2.1.2 **The Blood-CSF Barriers**

The CSF is actively produced in the brain ventricles by the choroids plexus (Figure 5.1A). The CSF then drains into the subarachnoidal space and is resorbed into the blood through the arachnoidal villi of the dura mater venous sinuses, and partly into the cervical lymphatic system via prolongations of the subarachnoidal space around the olfactory nerves [41]. The difference in composition between the blood and the CSF reflects the existence of a blood–CSF barrier which restricts the entry of blood components into the subarachnoid space in a similar manner to the blood–brain barrier. Two localizations of the blood–CSF barrier can be distinguished: the ventricular and the meningeal blood–CSF barriers (Figure 5.1C).

The choroids plexus (CPs) (Figure 5.1C) are highly vascularized villus structures localized in brain ventricles. The capillaries and venules of the CPs are naturally fenestrated and lack tight junctions, allowing a passive diffusion of blood cells and electrolytes into the CPs' stroma. The epithelial-like cells that delineate the CPs actively produce the CSF. These cells are linked to junctional complexes by tight junctions. They form the ventricular blood–CSF barrier.

The meningeal blood–CSF barrier is subdivided in two compartments: the subpial space, which is contiguous with the CNS, and the subarachnoidal space in which the CSF circulates (Figure 5.1C). As described above for the brain venous system, inter-endothelial tight junctions of subpial and meningeal veins form a somewhat more leaky structure compared to that of CNS capillaries [37]. The pia and arachnoid maters may also act as barriers to limit the spread of infection by bacteria that may have crossed the endothelial cell barrier. However, leptomeningeal cells form a thin monolayer structure that lacks tight junctions and probably does not provide an adequate barrier to the infectious process. Indeed, patients who die of purulent meningitis show inflammatory cells both in the subarachnoid spaces and in the contiguous subpial space suggesting that inflammatory cells can cross the pia mater [42]. Immune surveillance of the meningeal spaces is provided by F4/80 positive macrophages and MHC class II phagocytes that adhere to the pia and arachnoid mater and to the surface of the choroids plexus in the ventricles [43, 44].

In the newborn, the CSF contains a greater number of leukocytes and proteins than that in adults (up to 20 versus 5 leukocytes per mm^3 and up to 1.5 versus 0.18 to 0.58 $g\,l^{-1}$ of protein in newborns and adults respectively) [45–47]. Normal values are reached between 6 and 12 months of age. Experimental studies in animals have shown that tight junctions that restricts the paracellular passage of lipid-insoluble molecules through the blood–CSF barrier are functionally mature very early in development, suggesting that both proteins and passive markers are transferred across the blood–CSF barrier via a transcellular route [48–50]. During systemic inflammation, the blood–CSF barrier in the newborn might be less efficient at preventing the entry of activated leukocytes into the SAS than that in children or adults. Indeed, CSF pleocytosis without cultivable bacteria has been observed in the cerebrospinal fluid of young infants hospitalized for urinary tract infection [51–53]. An explanation for this observation may be that the blood–CSF barrier of the newborn has an increased reactivity to circulating bacterial components or systemic cytokines released during peripheral infectious processes.

5.2.2
The Passage of Extracellular Bacterial Pathogens across the Blood–CSF Barrier

5.2.2.1 Where is the Blood–CSF Barrier Crossed?

Experimental models of blood-borne meningitis initiated by *E. coli* [54], *S. agalactiae* [55], *H. influenzae* [54, 56], *S. pneumoniae* [9] and more recently *N. meningitidis* [57, 58] have been developed mostly to identify the virulence factors involved in bacterial survival within the blood and their passage across the blood–brain barrier. Alternatively, *in vitro* cell culture models using human brain-derived endothelial cell lines that express tight junctions have played a crucial role in determining how meningitic bacteria specifically interact with the endothelial blood–CNS interface [29, 59]. These models have led to the identification of bacterial adhesins and specific host ligands such as the platelet activating factor

receptor for *S. pneumoniae* [60, 61]. However, the precise site and mechanism of entry of extracellular bacterial pathogens into the CSF is still enigmatic.

As shown from post mortem examination [62], extracellular bacterial pathogens interact directly with the components of the blood–brain barrier and do not need a 'Trojan horse' such as leukocytes to cross the BBB. It has been postulated that bacteria may preferentially use the choroids plexus (CPs) route to cross the blood–CSF barrier. However, in this case, meningitis should theoretically be associated with ventriculitis, which is not corroborated by clinical and experimental data. Because of their proximity to the subarachnoid space and their 'leaky' inter-endothelial structure, post-capillary venules of the subpial and subarachnoid spaces in the brain are a likely site of entry into the CSF for bacterial pathogens. Indeed once the bacteria have crossed the endothelial monolayer of these vessels, they will be only separated from the CSF by a thin monolayer of leptomeningeal cells. Post-capillary venules of the parenchyma could also be involved since the Virchow–Robin perivascular spaces are continuous with the subpial space (Figure 5.1A).

5.2.2.2 How do Extracellular Bacteria Breach the Blood–CSF Barrier?

There are at least four possible strategies for a microorganism to cross a monolayer of endothelial (or epithelial cells) (Figure 5.2): bacterial adhesion to endothelial cells followed by (i) transcellular transport by passive or adhesion-induced transcytosis, (ii) paracellular passage through opened tight junctions, or (iii) disruption of the endothelial barrier due to a direct cytotoxic effect and (iv), leukocyte-facilitated transport by infected phagocytes (the Trojan horse mechanism). These routes are not exclusive as exemplified by virus entry into the CNS which takes place directly both by interaction with the blood–brain barrier or transportation by infected phagocytes [63]. As already mentioned, extracellular pathogens probably do not use leukocytes as vehicles to cross the blood–CSF barrier. A breakdown of the blood–CNS barrier due to apoptosis or bacterial cytotoxity is unlikely since it would be associated with lesions leading to hemorrhage in the subarachnoidal space, which is uncommon during bacterial meningitis. Therefore, the entry of blood-borne pathogens is unlikely to occur via the rupture of the blood–CNS barrier [9]. If this is the case then adhesion of bacteria to endothelial cells may induce intracellular signaling to initiate disruption of intercellular tight junctions or, alternatively, bacteria may induce their own transcytosis through the cell monolayer. *In vitro*, transcytosis of bacteria through human brain endothelial cells has been demonstrated for *S. pneumoniae* [64], *E. coli.* [65–67], and *S. agalactiae* [68], and through human umbilical vein endothelial cells for *H. influenzae* [69, 70]. Although *N. meningitidis* can readily be internalized within vacuoles in human brain microvascular endothelial cells [71], the route used by the meningococcus to cross the endothelial cells is still under debate [5].

As stated above, high grade bacteremia is required to induce bacterial meningitis by extracellular pathogens [72–74]. Although a high bacteremia probably enhances the opportunity for a pathogen to adhere to and cross the blood–CSF barrier, this observation may also suggest that activation of endothelial cells by circulating

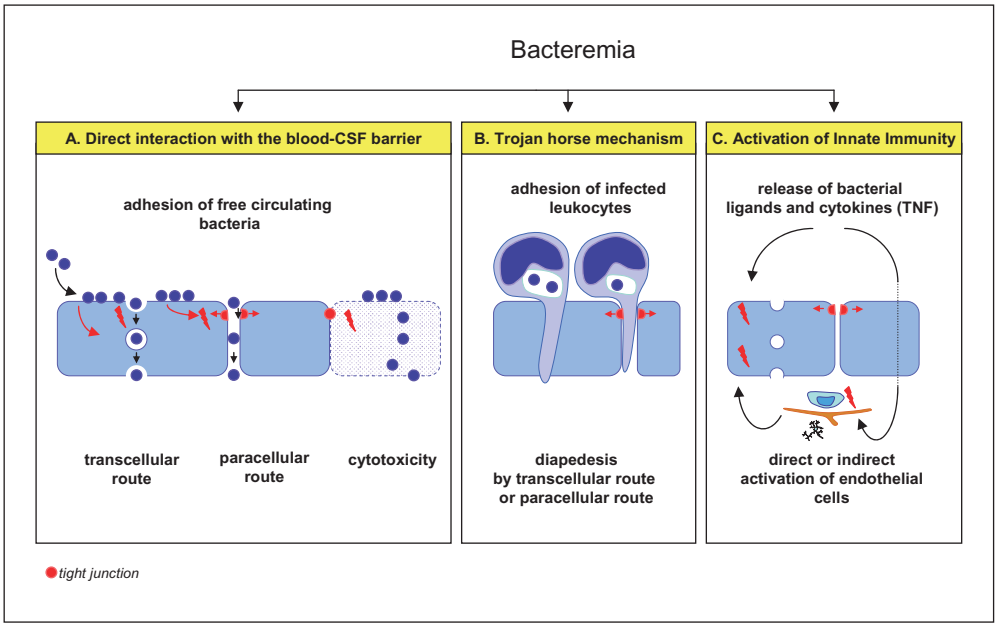

Bacteremia
A. Direct interaction with the blood-CSF barrier
B. Trojan horse mechanism
C. Activation of Innate Immunity
adhesion of free circulating bacteria
adhesion of infected leukocytes
release of bacterial ligands and cytokines (TNF)
transcellular route
paracellular route
cytotoxicity
diapedesis by transcellular route or paracellular route
direct or indirect activation of endothelial cells
tight junction

Figure 5.2 Possible mechanism of traversal by which extracellular blood-borne bacteria cross the blood–CSF barrier. Bacteria circulating in the bloodstream can either directly interact with endothelial cells (A) or cross the vessel within infected phagocytes: the Trojan horse mechanism (B). The latter hypothesis is doubtful for extracellular bacteria which are capsulated and resist phagocytosis. The first step of bacterial direct interaction with endothelial cells is adhesion. Bacteria can then induce their own transcytosis within vacuoles, a mechanism that is suspected for *H. influenzae*, *S. pneumoniae*, *E. coli* and *S. agalactiae*. Alternatively, adhering bacteria may induce a signaling cascade and weaken the interendothelial junctional complexes, thus opening a paracellular route to bacteria which multiply at the luminal face of the endothelial cell. Bacterial adhesion may also lead to endothelial cell death, either by direct cytotoxicity or induced apoptosis. The activation of innate immunity that is triggered by the invading pathogens at the onset of systemic infections could also favor bacterial adhesion and traversal of the blood–CSF barrier by increasing the expression of membrane ligands to bacterial adhesins on endothelial cells, or by increasing the permeability of the vascular barrier. Activation of endothelial cells could occur either directly by circulating bacterial molecules or indirectly via the synthesis of pro-inflammatory cytokines released into the blood or produced by perivascular cells such as astrocytes, perivascular macrophages or microglial cells. It should not be assumed that these putative mechanisms are exclusive.

bacterial compounds or cytokines released in response to the systemic infection may promote bacterial entry into the CSF (Figure 5.2). An emerging concept is that the innate immune response induced during bacteremia, although not leading to septic shock, may unfortunately favor bacterial meningitis by increasing the permeability of the blood–CNS barrier to meningitic bacteria. Various bacterial components can rapidly activate the cellular components of the innate immune system after their entry into the body. Experimental models of hematogenous

meningitis and bacteremia have shown that in spite of the diversity of the signaling pathways induced by meningitic bacteria, the early inflammatory response is somewhat similar [75–77], particularly involving tumor necrosis factor alpha (TNFα), interleukin-1β (IL1-β) and IL6 which are also key mediators of septic shock [78]. Interestingly, the peak of early pro-inflammatory cytokines corresponds experimentally to the peak of bacteremia and to the onset of bacterial invasion of the meningeal spaces.

Evidence that severe bacterial infections can alter the permeability of the blood–brain barrier without brain infection was first demonstrated clinically in the septic encephalopathy syndrome. Although the etiology of this syndrome is unclear, it involves cerebral edema and disruption of the blood–brain barrier that may arise from the action of circulating bacterial products or pro-inflammatory cytokines [79, 80]. TNFα reversibly increases the transcellular permeability of brain microvascular endothelial cells [81] and has been shown to be one of the key mediator of this disease in an experimental model of LPS-induced septic encephalopathy [82]. *In vitro*, TNFα induces the expression of the G-protein-coupled platelet activating factor (PAF) receptor that is required for the efficient adhesion to and passage across human brain endothelial cells by *S. pneumoniae* [61]. Microvascular brain endothelial cells can also release TNFα upon stimulation by cell wall components of *S. pneumoniae* and can thus be activated with via an autocrine loop [83]. Many humoral compounds such as cytokines, bacterial compounds [72] or other humoral factors such as histamine, reviewed elsewhere, [84, 85] can activate endothelial cells and increase BBB permeability. This may occur either by direct activation of endothelial cells as stated above or via the activation of CNS perivascular cells. Indeed, there is increasing evidence to suggest that endothelial cells can transmit blood danger signals to the brain either by transcytosis of circulating pro-inflammatory compounds or indirectly by their own activation which in turn induces the release of a downstream mediator such as prostaglandin E2 into the perivascular space [86–89]). Finally, brain perivascular cells, including perivascular macrophages, microglial cells and astrocytes in the brain parenchyma express toll-like receptors and can produce various cytokines and chemokines, thus in turn activating the endothelial cells and altering the permeability of the vessel [90–94].

Recently, an elegant demonstration of the role of pro-inflammatory cytokines in the entry of microorganisms into the CNS has been reported using West Nile virus encephalitis as the experimental model[95]. In this study, the authors showed that, although toll-like receptor 3 (TLR3, a ligand of double-stranded RNA) was required to control systemic infection, TLR3-deficient mice were more resistant to lethal West Nile virus infection and showed lower neuropathology in comparison to wild-type mice. TLR3 signaling particularly induced the synthesis of TNFα, IL1 and interferon β, and the peak of TNFα corresponded to the time of entry of the virus into the CNS. Focusing on TNFα signaling, the authors demonstrated that TNFα receptor 1 was involved in the disruption of the blood–brain barrier upon TLR3 stimulation by double-stranded RNA. *In vitro*, TNFα signaling has also been implicated in the entry of HIV-1 into both endothelial and trophoblastic cells in the brain [96, 97].

5.3 Neonatal Meningitis

The World Health Organization lists meningitis among the five leading causes of neonatal infections worldwide. Each year, in developing countries, about 50 000 newborns die of meningitis (mortality rate 40%), representing about 1% of all causes of neonatal mortality [98]. In industrialized countries the incidence of neonatal bacterial meningitis is between 0.2 and 0.4 per 1000 live births [99–103], with an estimated mortality rate of 15 to 30% [101, 102, 104] which rises to nearly 40% in premature infants. In fact, group B streptococci (GBS) and *Escherichia coli* account for 70 to 80% neonatal bacterial meningitis cases in industrialized countries [105]. *E. coli* is currently the second cause of neonatal meningitis, behind GBS, however in the subpopulation of premature infants, *E. coli* is the leading pathogen [106]. In developing countries, GBS meningitis is far less frequent than meningitis caused by *Enterobacteriaceae* [98].

5.3.1 *Escherichia coli* Meningitis

Most cases of *E. coli* meningitis occur during the first months of life and adult cases are exceptional. The vast majority of these infections occur during the neonatal period (<28 days), and only 10 to 20% of cases of *E. coli* neonatal meningitis occur between 1 and 3 months of age [104, 107]. The sequence of events leading to meningitis is as follows. Following acquisition from the mother's flora or from the environment, pathogens colonize the infant intestinal tract. The development of meningitis then comprises three main steps. The first step is bacterial translocation to the bloodstream, usually originating from the intestinal lumen [108, 109]. Alternatively, in up to 20% of *E. coli* neonatal meningitis cases, the port-of-entry is a urinary tract infection [107]. The second step is a sustained high-level bacteremia due to intravascular survival and multiplication of the organism [74, 108, 110]. The third step is characterized by the passage of bacteria through the blood–CSF barrier and invasion of the arachnoidal space. As mentioned above, this last step is associated with critical blood bacterial density thresholds. Experimental and clinical studies have shown that bacteremia below or equal to 10^4 CFU/ml leads to meningitis in 10% of cases, while bacteremia above 10^6 CFU/ml leads to meningitis in at least 80% of cases [110, 111].

5.3.1.1 *E. coli* Strains Causing Neonatal Meningitis (ECNM) Are Oligoclonal

E. coli species has a clonal structure and is organized into four main phylogenetic groups designated A, B1, B2 and D [112]. Extra-intestinal pathogenic *E. coli* (ExPEC) have an oligoclonal distribution and mainly belong to group B2 and D. Serotyping of *E. coli* neonatal meningitis strains during the 1970s and 1980s suggested a strong oligoclonality among ECNM, as nearly 90% of the strains had the capsular serotype K1 which was associated with antigens O18 and O7 in 50% of cases [113, 114]. In addition to these historical clones O18:K1:H7 and O7:K1, new serotypes

have recently emerged such as O83:K1 in Netherlands and O45:K1:H7 in France [115, 116]. A recent molecular epidemiological investigation using multi-locus sequence typing (MLST) has definitively demonstrated that ECNM strains have the most marked oligoclonal structure among ExPEC strains [117]. Of the 186 ECNM strains isolated in Europe and North America 86% were found to be present in only seven of the hundreds of sequence type complexes (STc) stored in the *E. coli* MLST database (http://www.shigatox.net). More spectacularly, 50% of isolates belonged to only one STc of the B2 group (STc29), and this STc encompassed O18:K1 and O45:K1 strains [117]. The combination of STc and the serogroup designated 'Sequence-O-type' allowed a more precise definition of the seven major clonal subgroups which cause neonatal meningitis: STc700-O1 (n = 10), STc301-O7 (n = 13), STc304-O16 (n = 12), STc697-O83 (n = 11), STc29-O1 (n = 8), STc29-O18 (n = 48) and STc29-O45 (n = 39). These molecular epidemiological studies demonstrated that ECNM strains belong to a few clonal complexes that have acquired the ability to invade the subarachnoidal space in infants.

5.3.1.2 The Specific Virulence Determinants of ECNM Strains

The search for genetic determinants involved in each step of the pathogenesis of ECMN has been facilitated by several *in vivo* and *in vitro* models [118–120]. Using these models several strategies have been implemented such as transposon mutagenesis [121], representational difference analysis [122], signature tagged mutagenesis [123–125], differential fluorescence induction [126], *in silico* comparative genomic analysis [127] and finally genome sequencing [128, 129]. Among the virulence determinants identified, some of them belong to the core genome and are therefore present in almost all *E. coli*. We will concentrate on these specific virulence genes, i.e. genes not found in the core genome, which have been incriminated in ECNM pathogenesis.

Although some genetic determinants of the *E. coli* strains that colonize the gastrointestinal tract have been identified [125], the molecular mechanisms involved in the intestinal translocation step remain largely undefined. Recently Fagan *et al*. characterized a protein named Hek that promotes adhesion to and invasion of gastrointestinal cells [130]. However, this gene is present in only 10% of ECNM strains [115].

In the clonal group O18:K1:H7, four specific determinants have been recognized to contribute to the high level of bacteremia during neonatal meningitis. (i) The K1 capsule may be considered as one of the most important. This capsule is present in up to 80 to 90% of ECNM strains and in only 30% of strains isolated from the stools of healthy patients. The K1 antigen is composed of a homopolymer of α2–8 acetyl neuraminic acid (sialic acid), which is identical to that of serogroup B *Neisseria meningitis*. The major role of the capsule is to allow intravascular multiplication and survival of the bacteria. Kim *et al*. have shown that the level of bacteremia falls dramatically in the presence of an uncapsulated mutant compared to that which occurs in the presence of the wild type parental strain [110]. Two main properties of the polysialic acid may explain why the K1 capsule is crucial to the virulence of ECNM strains. This antigen is recognized as a self-antigen,

since it is a physiological component of the neuronal fetal N-CAM [131], therefore in the absence of specific antibodies there is no activation of the classical complement pathway by the K1 antigen. Moreover K1 has a high affinity for the H factor, a strong inhibitor of the C3 convertase and may subsequently block the alternate complement pathway [132]. The O18 lipopolysaccharide is also considered to be a protectin, blocking the activation of the complement via the antibody-independent classical pathway [133]. (ii) Other specific traits of ECNM strains are the iron uptake systems. The siderophore salmochelin is present in two-thirds of ECNM strains. Other siderophores such as yersiniabactin or aerobactin are present in 100 and 86% of ECNM strains, respectively [115]. However salmochelin remains the sole iron chelation system known to play a role during the bacteremic step of meningitis [129, 134]. (iii) A pathogenicity island (PAI) initially described in the representative strain C5 (O18:K1:H7), and named PAI I_{C5} [111] is also specific to ECNM. This PAI, ~100 kb long, resembles the PAI II_{J96} of the uropathogenic strain J96 [135], harboring several putative virulence factors including the P fimbriae, hemolysin, cytotoxic necrotizing factor-1 (CNF-1) and the invasin Hek cited above. Although hemolysin probably plays a role in the virulence of PAI I_{C5}, the exact mechanism by which this ectochromosomal DNA is involved in the highly-sustained bacteremia remains unknown. This PAI is present in only 10% of the European O18:K1:H7 strains and is only exceptionally found among other clonal groups. (iv) Recently a new major genetic determinant, a plasmid of 133 kbp, responsible for the high level of bacteremia, has been identified and sequenced in S88, a representative strain of the O45:K1:H7 clonal group (www.genoscope.cns.fr). Interestingly, this plasmid is present not only in all the O45:K1:H7 strains but also in most of the O18:K1:H7 strains devoid of a PAI similar to PAI I_{C5} and therefore may lead to the identification of new virulence factors.

The availability of *in vitro* BBB models composed of human brain microvascular endothelial cellss (HBMEC) has led to the identification of factors involved in the transcytosis of *E. coli*. Two major factors play a crucial role in the initial interaction with the host cells and are encoded by genes present in most commensal *E. coli* isolates. These includes the type 1 fimbriae with the FimH adhesin which recognizes mannose residues [136] and the Outer Membrane Protein A, OmpA, which binds through N-glucosamine epitopes of gp96 [137]. The invasion of HBMEC occurs via a zipper like-mechanism and *E. coli* are found internalized within membrane-bound vacuoles in HBMEC examined using transmission electron microscopy [138]. Several specific virulence factors that are involved in this step of the interaction between bacteria and their host receptors have been identified and include invasin IbeA and a 45-kDa protein [63] or the CNF-1 and the 37-kDa laminin receptor precursor [139]. Once internalized, the K1 capsule has been shown to be essential for intracellular survival. The mechanism by which the K1 capsule modulates the maturation process of *E. coli* K1-containing vacuoles in HBMEC remains to be elucidated [140].

Although *E. coli* is the main Gram negative pathogen responsible for bacteremia in adults, *E. coli* meningitis is unusual in adult patients. This puzzling paradox is not explained by differences in the structure and/or anatomy of the brain

vasculature between neonates and adults [67]. A likely explanation for this apparent paradox is the level of bacteremia which is much higher in neonates than in adults. Indeed quantitative blood cultures in adults have shown that bacteremia never exceeds 1 to 10 bacteria/ ml and therefore does not reach the threshold level of 10^3 to 10^4 bacteria/ml, a *sine qua non* condition for meningeal invasion by *E. coli*. The functional immaturity of the neonatal immune system is likely to be responsible for the ability of these extracellular pathogens to multiply in the bloodstream. Complement levels and activity are significantly reduced in neonates compared to the levels in adults and C8 and C9 are the most markedly reduced complement components [141]. Lassiter *et al*. convincingly showed that administration of complement component C9 enhances the survival of neonatal rats infected with *E. coli* [142]. In addition, other components of innate immunity are deficient in neonates, such as the bactericidal/permeability-increasing protein in the polymorphonuclear neutrophils [143], and the expression of toll-like receptor 4 and cytokines in response to lipopolysaccharide stimulation [144].

5.3.2 Meningitis Due to Group B *Streptococcus* (GBS)

Group B streptococcus (*Streptococcus agalactiae*) is a Gram-positive encapsulated bacterium and a physiological constituent of the intestinal flora in humans. It is also present in the vaginal flora of 30% of healthy women. However, it is a deadly pathogen in neonates for whom it is the leading cause of pneumonia, septicemia, and meningitis. Meningitis can lead to significant morbidity, 25–50% of surviving infants suffer neurological sequelae of varying severity, including cerebral palsy, mental retardation, deafness, or seizures [145]. In neonates, there are two types of GBS disease: the early onset disease (EOD) defined as occurring within the first week of life and the late onset disease (LOD) which occurs after the first week of life. EOD GBS infection often develops in term neonates with no defined risk factors other than colonization. However, premature onset of labor and chorioamnionitis are proven risk factors for EOD GBS infections [145].

In the case of EOD, bacterial contamination occurs either *in utero* through the placental membranes or during parturition by inhalation of GBS-contaminated vaginal or amniotic fluid. The risk of being colonized by the vertical route correlates directly with the intensity of the colonization (inoculum size). As a consequence, pneumonia can develop and the bacteria can disseminate into the bloodstream and invade the subarachnoid space. In the case of LOD, meningitis is the most frequent clinical manifestation (>50% of the cases), however, the mechanisms by which newborns are contaminated is unclear.

5.3.2.1 GBS Strains Responsible for Neonatal Meningitis Are Oligoclonal

A substantial proportion of EOD and the majority of LOD have been correlated worldwide with one of the 10 known capsular serotypes, serotype III [146–150]. Serotype III contains a limited number of clonal complexes, defined by MultiLocus Sequence Typing (MLST), among which ST-17 is overrepresented. Indeed, the

ST-17 clone accounts for the vast majority of neonatal invasive diseases and for almost all cases of GBS meningitis and was therefore designated as the 'hypervirulent clone' [146, 147, 151]. This overrepresentation of ST-17 among invasive neonatal strains is now well recognized worldwide and highlights the fact that this clone is well adapted to neonate pathogenesis and may possesses specific virulence traits that enhance its invasiveness in children of this age.

5.3.2.2 The Specific Virulence Determinants of GBS Strains Responsible for Meningitis

Studies in humans and animals have shown that a high bacteremia is required for the development of meningitis, as observed for almost all extracellular pathogens responsible for meningitis [152]. However, the precise mechanism(s) whereby GBS leave the bloodstream and gains access to CNS remains to be determined.

The development of GBS meningitis implies that once in the bloodstream, bacteria survive and multiply by overriding host phagocytic cells including polymorphonuclear neutrophils and macrophages. GBS then adheres to microvessels in the brain, and invades and/or transcytoses them to reach the CNS. *In vitro* studies demonstrated that GBS efficiently invaded hBMECs and survived intracellularly [68]. Transmission electron microscopy showed that GBS adheres tightly to the cell surface and can be internalized within a membrane-bound endocytic vacuole [68]. Although the role of several virulence factors in the GBS infectious process has been described, only a few determinants have been proven to impact directly or indirectly on the pathogenesis of meningitis [145, 153]. Among these virulence determinants, the surface capsular polysaccharide (CPS) has been studied in greatest detail. Almost all GBS involved in human diseases are encapsulated. The serotype-specific epitopes of each polysaccharide are created by various arrangements of four sugars (glucose, galactose, *N*-acetylglucosamine and sialic acid) into a single repeat unit. The presence of a sialic acid (Neu5Ac) bound to galactose in an alpha2→3 linkage is a constant feature of the Group B streptococcal capsular polysaccharides. This GBS terminal alpha2→3 Neu5Ac capsular component is identical to a sugar epitope widely displayed on the surface of mammalian cells [154]. The main role of CPS is to protect GBS by avoiding opsonophagocytosis. Isogenic uncapsulated mutants are more susceptible to killing by human neutrophils and exhibit a 100-fold increase in LD50 values as compared to the wild type strain in a neonatal rat model [155]. CPS and in particular the sialic acid residues, inhibit the alternative complement pathway by blocking binding of C3 to the organism and promoting inactivation of C3b. Several animal models provided evidence that GBS CPS protects the organism against phagocytic clearance during both the initial pulmonary phase and the later bacteremic phase of EOD.

The GBS β-hemolysin/cytolysin (β-h/c), encoded by the *cyl* locus, is a pore-forming toxin which is present in most human GBS strains. At this locus, *cylE*, encodes a 79-kDa putative protein that confers hemolysin–cytolysin expression on GBS which shares no homology with streptolysin S or other bacterial hemolysins identified so far [156]. Another unique feature displayed by GBS β-h/c is that it is linked to the production of an orange pigment, recently identified as granadaene

[157]. GBS β-h/c has not only been implicated in lung epithelial cell injury but also in brain microvascular cell injury. GBS β-h/c mutants show decreased BBB penetration and decreased lethality from meningitis in a mouse model of infection [158].

Other virulence factors have been directly implicated in the disease process of meningitis. As GBS adhesion and invasion of hBMECs constitutes a crucial step in the pathogenesis of neonatal meningitis, various surface components have recently been reported to interact and impact on the process of passage across the BBB. A mutant of *iagA*, a gene encoding a glycosyltranferase involved in the appropriate cell-surface anchoring of lipotechoic acid, exhibited decreased adhesion and invasion of hBMECs and did not produce meningitis in mice [55]. A likely explanation suggested that loss of the cell membrane glycolipid anchor LTA (DGlcDAG) removed a potential 'invasin' from the GBS surface. *In vitro* experiments demonstrated that FbsA, a fibrinogen-binding protein and Lmb, a laminin-binding protein, promoted adhesion and invasion to hBMECs [159, 160]. Cell surface-expressed filamentous appendages known as pili have recently been identified in streptococcal pathogens that cause invasive infections in humans, including GBS [161–163]. Pili are often involved in bacterial adherence to host cells and tissues during colonization. The analysis of multiple GBS genomes has revealed the presence of specific genetic islands that contain the necessary components for pilus formation [161, 162]. In all cases that have been described so far, the genes that encode the pilus proteins are clustered at the same genetic locus, transcribed in the same direction, and likely part of an operon. The pilus operon codes for cell wall-anchored LPXTG proteins and class C sortases which are required for pilus assembly [161, 162]. Recent studies strongly suggest that GBS pili in strain NEM316 are composed of three precursors LPXTG proteins: Gbs1477 (PilB), the *bona fide* pilin which is the major pilus component; Gbs1474 (PilC), a minor pilus-associated component that may anchor the pilus to the cell wall, and Gbs1478 (PilA), the pilus-associated adhesin [161]. However, the exact mechanism by which these structures facilitate adhesion and invasion as well as their putative cellular host ligand remains to be determined [164].

5.4 Cerebrospinal Meningitis

Neisseria meningitidis is the etiological agent of cerebrospinal meningitis, which occurs mostly frequently in children under 1 year of age and in young adults. This disease is still responsible for over 30 000 deaths worldwide annually [1]. Paradoxically *Neisseria meningitidis* is a frequent asymptomatic colonizer of the human nasopharynx, and only a very small proportion of infections proceed to sustained bacteremia and thence to meningitis or septicemia. The reasons why disease occurs in some individuals and not in others remain unclear, but human factors are likely to be important in determining the outcome of infection. The most important factors predisposing individuals to disease are the absence of bacteri-

cidal antibodies and dysfunction of the complement system. Some polymorphisms of the TLR4 receptor have also been associated with meningococcal disease. In addition, not all meningococci are associated with the same pathogenic potential. Indeed, the analysis of results from multilocus sequence typing (MLST) has demonstrated the existence of distinct phylogenetic groupings (clonal complexes), some of which are consistently more likely to be isolated from patients than are others. These are the so-called hyper-virulent or hyperinvasive lineages. The presence of a prophage in strains belonging to hyperinvasive lineages has recently been shown to be responsible for their high level of invasiveness[165]. In addition, there is a clear association between the presence of this prophage and virulence in young adults but not in children under the age of 2 years. This element which is inserted into the chromosome can be induced to produce a filamentous phage. Experiments attempting to identify the phenotype encoded by the phage that is responsible for hyperinvasiveness have not been conclusive. Indeed deletion of the entire prophage did not have any effect in laboratory models of meningococcal pathogenesis. Several observations suggest that this prophage is associated with genomic rearrangements. These modifications may be associated with an increased fitness of the bacteria for dissemination in the bloodstream.

Pathology is initiated when the bacteria reach the bloodstream. Very surprisingly, in some patients bacteria are cleared from the bloodstream and the only symptom is a febrile flu-like syndrome. When bacteria are not cleared from the blood, two main manifestations may occur. The first is a fulminant septicemia that has a mortality rate that varies between 20 and 30%. This disease is characterized by rapidly evolving septic shock and disseminated intravascular coagulation. Skin hemorrhages (purpura fulminans) and a large number of replicating bacteria in the organs are the hallmark of this form of disease. Although bacteria cross the blood–CSF barrier during the course of fulminant septicemia, the manifestation of inflammation does not occur during this brief time period. The second more classical event following invasion of the bloodstream by *N. meningitidis* is the passage of bacteria across the BBB and the initiation of non-specific inflammation in the subarachnoidal space. As already mentioned, it has been estimated that 63% of cases of bacteremia due to *N. meningitidis* are associated with meningitis, whereas only 9% of cases of pneumococcal bacteremia are associated with meningeal invasion. This high specificity is a consequence of both the adaptation of bacterial growth to human extracellular fluid and the tight associations created between the bacterial cells and the endothelial cells of the capillaries.

As for neonatal meningitis it is believed that the level of bacteremia is directly correlated with meningeal invasion. The bacterial attributes involved in their growth and/or survival in extracellular fluids therefore play an essential role in meningeal invasion by *N. meningitidis*. Some of these virulence factors are common to most extracellular pathogens and are capable of preventing killing by the effectors of the innate immune systems such as polymorphonuclear neutrophils and complement. These attributes are the polysaccharidic capsule, the lipooligosaccharide, and the iron chelation systems and have been the subject of numerous reviews. Recently a new virulence factor has been identified, the factor-H binding

protein (fHBP) [166]. The latter is a 28-kDa surface-exposed lipoprotein that binds factor H, a key inhibitor of the complement alternative pathway. This protein is expressed by all *N. meningitidis* strains studied to date, however the level of expression varies between strains (high, intermediate, or low expressers). Antibodies against fHBP are bactericidal and this protein is one of the best vaccine candidates [167]. Bacteremia is believed to result from meningeal invasion by directly increasing the likelihood of interaction between the bacteria and components of the BBB. However, it should be pointed out that the role of the innate immune effectors in the breach of the BBB remains unknown.

Very few septicemic extracellular bacterial pathogens have the ability to form such tight interactions with the endothelial cells as *N. meningitidis*. Indeed, post mortem examination of a case of fulminant meningococcemia clearly demonstrated that *N. meningitidis* freely interact as extracellular pathogen with the endothelial cells of the skin, kidney, spleen, lever and brain [62, 168]. The interaction of *N. meningitidis* with the vessels of the brain parenchyma plays an important role in the invasion of the meninges. More generally, the widespread adhesion of *N. meningitidis* to vessel walls throughout the body may be responsible for the loss of integrity of the vessels seen in fulminant meningococcemia and for the extravasation of red blood cells responsible for the petechies in less severe cases.

Blood flow generates mechanical forces that may vary depending on the type of vessels and that could prevent bacterial interaction with the endothelial cells. The ability of *N. meningitidis* to bind to endothelial cells in the presence of a flow of liquid mimicking the bloodstream was recently investigated [62]. These data revealed an adhesion mechanism and the results showed that after the initial attachment process the bacteria were able to resist high blood velocities, multiply, and form microcolonies. This resistance to shear stress and the ability to grow onto the endothelial cells during blood flow underline the efficiency of the interaction between *N. meningitidis* and the host cells. In all cases, *N. meningitidis* has developed very efficient mechanisms to ensure effective and secure binding to the endothelial cells. Furthermore, following this interaction, the bacterium generates a signal to facilitate its passage across the endothelial monolayer leading to meningeal invasion. Both events i.e. adhesion and signaling are promoted by the type IV pili.

5.4.1 Meningococcal Attributes that Allow Blood-borne Bacteria to Interact with Endothelial Cells

Various bacterial attributes which facilitate the interaction of *N. meningitidis* with human cells have been described. These are type IV pili and other attributes such as Opa proteins, Opc and more recently the secreted component of a two-partner secretion system. However in capsulated bacteria only type IV pili promote bacterial adhesion since non-piliated capsulated bacteria are unable to adhere to any type of cell. Early work undertaken with piliated capsulated meningococci has shown that meningococcal interaction with human cells can be divided into two steps. The first is the adhesion of single diplococci, in a rather inefficient process. The second step is bacterial division onto the apical surface of the cells. Therefore

the high number of bacteria that interact with cells is a consequence of the division of the few meningococci which initially succeeded in adhering to the host cells. The type IV pili are required for these two steps because they first promote the initial interaction of diplococci with the endothelial cells and they then generate bacterial interactions thus leading to the spread of the bacteria over the cell surface.

Type IV pili are polymeric filaments found on many Gram-negative bacteria. These structures correspond to a multimer of a pilin subunit protein encoded by the *pilE* gene. The pilin subunit (PilE) is synthesized as a pre-protein, and is produced by cleavage of an N-terminal sequence from prepilin by a prepilin peptidase PilD [169]. Fully functional Tfp are dynamic structures. Pilin subunits are constantly being assembled into fibers from a plate-form in the inner membrane. The fiber is then extruded through the outer membrane. A major characteristic of Tfp is their ability to retract. Retraction is a consequence of the disassembly of pilin subunits that are then stored in the cytoplasmic membrane. A summary of the various bacterial components necessary for pilus biogenesis is shown in Figure 5.3.

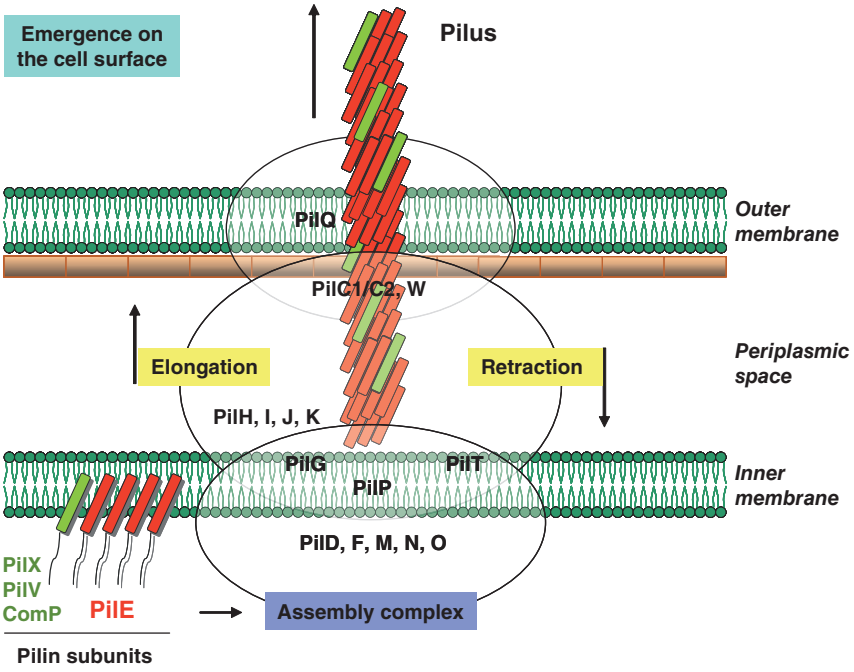


Figure 5.3 Type IV pilus biogenesis. At the inner membrane–periplasm interface, the pilin subunits (PilE) are assembled from a plate-form complex (PilD, F, M, N, O, P) and the growing fiber is translocated to the cell surface via the secretin (PilQ). PilP co-purifies with the inner membrane and interacts with PilQ. Two inner membrane ATPases promote pilus elongation with PilF and pilus retraction with PilT. At the periplasm–outer membrane interface, different components are required to counteract pilus retraction (PilC1/C2, G, H, I, J, K W). PilC1/C2 and PilW are located at the outer membrane whereas PilG is contained in the inner membrane. Some of the minor pilins are incorporated into the fiber (PilV, X and ComP).

The nature of the components of the Tfp that interact with eukaryotic cells remains uncertain. Initial studies suggested that the PilC1 protein was the adhesin. This hypothesis relied mostly on the fact that PilC1 was required for pili to become adhesive. Since then a similar phenotype has been observed for numerous other pili components (see Figure 5.3), and the nature of the bacterial components that bind to host cells remain unknown. On the other hand, adhesiveness has been correlated with the ability to establish bacteria–bacteria interactions. These interactions are required to allow the initial adhering bacteria to grow as a colony on the apical surface of the cells. Some variants of pilin are associated with hyperadhesiveness whereas others are responsible for a low adhesive phenotype. Strong adhesiveness has been correlated with the ability of pilin to form bundles that connect bacteria together. A second component promoting bacteria–bacteria interactions is PilX, a minor component of the fiber that is assembled within the fiber in a similar manner to pilin. Recent data support a model in which surface-exposed motifs of PilX stabilize bacteria–bacteria interactions via homotypic interactions between PilX molecules from pili of different bacterial cells [170, 171]

The proposed host cell receptor for the type IV pili of *N. meningitidis* and the closely related pathogen *N. gonorrhoeae*, is CD46 or membrane cofactor protein (MCP) [172]. Mice expressing human CD46 were more susceptible to systemic challenge by *N. meningitidis* than were control animals, although this was independent of the piliation status of the infecting bacterium [173]. However, recent data support the notion that pilus-mediated infection occurs in a CD46-independent manner. Indeed, a specific down-regulation of CD46 expression in human epithelial cell lines by RNA interference did not alter the binding efficiency of piliated gonococci [174]. These data therefore call into question the function of CD46 as an essential pilus receptor for pathogenic Neisseriae and therefore suggest that there is another, as yet undefined, pilus receptor.

5.4.2
Signaling Triggered by *N. meningitidis* that Leads to the Extravasation of Bacteria through the Brain Vessels

As mentioned above, after their initial attachment to host cells, encapsulated meningococci proliferate, forming a local colony at their site of attachment on the cell surface. The formation of these colonies is driven by bacteria–bacteria interaction mediated by the pili. When adhering to the apical membrane of epithelial cells, *N. meningitidis* induces the local elongation of microvilli towards the bacteria, leading to their engulfment and internalization of a small proportion of adhering bacteria [175, 176]. Adhesion of *N. meningitidis* to endothelial cells promotes the local formation of membrane protrusions reminiscent of epithelial microvilli structures that surround bacteria and initiate their internalization within intracellular vacuoles [177]. Interestingly, the formation of such protrusions was also observed *ex vivo* by transmission electron microscopy analysis of brain sections from a child who died from fulminant meningitis [5]. These observations strongly suggest that such morphological modifications of the host cell membrane

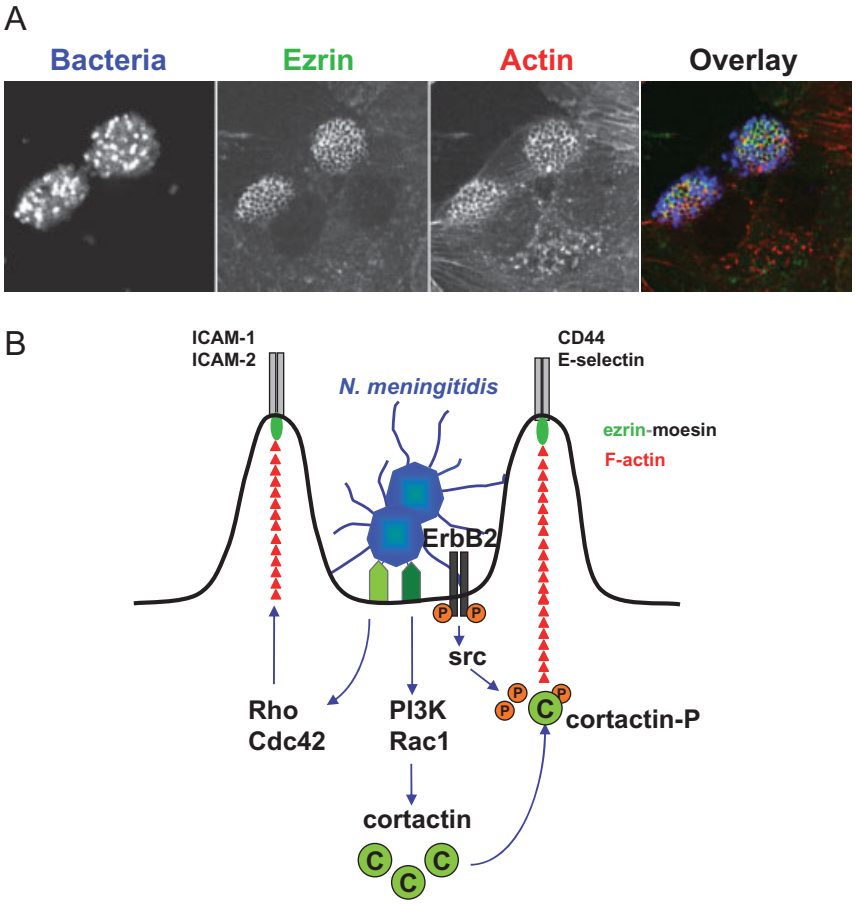


Figure 5.4 Infection of human endothelial cells by *Neisseria meningitidis* induces the formation of cortical plaques beneath bacterial colonies. (A) Immunofluorescence and confocal microscopy of human endothelial cells infected for 3 h with *N. meningitidis* (bacteria are in blue, ezrin in green and actin in red). (B) Schematic representation of the signaling pathways triggered by *N. meningitidis* leading to the formation of the cortical plaque.

may be essential for the passage of *N. meningitidis* across the human vascular endothelium.

The formation of membrane protrusions by encapsulated *N. meningitidis* originates from the organization of specific molecular complexes beneath the bacterial colonies referred to as cortical plaques (Figure 5.4). Cortical plaques result from the recruitment of the molecular linkers ezrin and moesin which cluster together several integral membrane proteins such as CD44 or ICAM-1 [177–179]. Ezrin and moesin control the organization of the cortical cytoskeleton by acting as linkers between the plasma membrane and the actin cytoskeleton. ERM proteins interact

via their amino-terminal domain with the cytoplasmic domain of transmembrane proteins (so-called ERM binding proteins), such as CD44 or ICAM-1, and with F-actin via their carboxy-terminal domain. The role of these molecules in *N. meningitidis*-induced cytoskeletal modifications has been demonstrated [177].

Like other pathogenic bacteria, *N. meningitidis* requires the participation of the actin cytoskeleton in order to penetrate non-phagocytic host cells. Cortical actin polymerization induced by *N. meningitidis* relies on the activation of both Rho and Cdc42 Rho GTPases, along with the activation of a PI3-K/Rac1GTPase signaling pathway involved in cortactin recruitment at the bacterial entry site [177, 180]. Cortactin is an actin binding protein involved in the reorganization of the cortical actin cytoskeleton. The recruitment and phosphorylation of cortactin at the *N. meningitidis* entry site is required for the formation of the actin-rich cell projections which promote efficient bacterial uptake, since the inhibition of either cortactin translocation or phosphorylation leads to structurally altered cortical actin polymerization [180]. Interestingly, tyrosine phosphorylation of cortactin induced by *N. meningitidis* results from the clustering and activation of the host cell tyrosine kinase receptor ErbB2 and the downstream activation of the kinase src [181]. ErbB2 belongs to the family of epidermal growth factor (EGF) receptors and the interaction of *N. meningitidis* with human endothelial cells leads to the activation of ErbB2, most likely via formation of ErbB2 homodimers.

The above signaling is observed only with piliated bacteria, thus demonstrating that pili are required for these rearrangements to occur. Furthermore this signaling is greatly enhanced when mechanical force is exerted on the host cell membrane. The force exerted by pilus retraction is thought to be the motive force that induces elongation of microvilli [182]. It is interesting to note that the signaling pathways promoted by *N. meningitides* and summarized in Figure 5.4, are similar to those induced by leukocyte adhesion to endothelial cells. Leukocyte adhesion promotes the remodeling of the apical endothelial plasma membrane into projections that surround adherent leukocytes [183–185]. These structures, referred to as 'endothelial docking structures' or 'transmigratory cups', are essential to promote firm adhesion and extravasation of leukocytes through paracellular as well as transcellular routes. These docking structures result from the dynamic redistribution of ICAM-1, VCAM-1 and CD44 at the endothelial–leukocyte contact area, accompanied by the recruitment of activated ERM proteins and by polymerization of cortical actin. Therefore, the same set of endothelial proteins is present in the membrane protrusions induced by *N. meningitidis* and in the docking structures promoted by leukocyte adhesion. In addition, by inducing the massive recruitment of ezrin and moesin together with ICAM-1, ICAM-2, VCAM-1, CD44, *N. meningitidis* colonies on the endothelial surface prevent their accumulation at leukocyte/endothelial cell contact areas and the formation of docking structures [186]. These data strongly suggest that when adhering to endothelial cells, *N. meningitidis* hijacks the cellular machinery normally used for leukocyte transendothelial migration to promote the formation of the cortical plaque.

The mechanism by which the bacteria cross the brain vessels following the formation of the cortical plaque is not well understood. Two different routes are

possible, the first is transcytosis followed by exocytosis at the basolateral surface. In favor of this hypothesis is the fact that a small proportion of the bacteria are internalized and data that have been obtained *in vitro* using a monolayer of epithelial cells forming tight junctions which show that the bacteria can cross such a monolayer without destroying the intercellular junctions. However, epithelial cells may not behave like endothelial cells and it cannot be excluded that in brain endothelial cells the formation of the cortical plaque induces the delocalization of intercellular junction proteins and the subsequent opening of the paracellular route.

Recent exciting findings have considerably expanded our understanding of the cellular events involved in *N. meningitidis* invasion processes, underlining the complex sequence of signaling events induced by *N. meningitidis* to elicit its uptake into non-phagocytic cells. However, in spite of recent advances in our understanding of these molecular mechanisms, much remains to be discovered about the complex molecular networks involved. Among the major issues is the identification of the receptor for meningococcal pili, which would constitute a significant breakthrough in the field and pave the way for future developments of novel vaccine strategies. However considering that pathogenesis is an accident and is not the usual outcome of *N. meningitidis* colonization of a host, one question remains to be answered, how does cellular invasion help this bacterium to disseminate? It is likely that invading host cells at the site of colonization may help *N. meningitidis* to persist inside its host and to evade the innate immunity of the mucosa.

References

1 Van de Beek, D., de Gans, J., Tunkel, A.R., and Wijdicks, E.F. (2006) Community-acquired bacterial meningitis in adults. *N. Engl. J. Med.*, **354**, 44–53.

2 Huang, S.H., Stins, M.F., and Kim, K.S. (2000) Bacterial penetration across the blood–brain barrier during the development of neonatal meningitis. *Microbes. Infect.*, **2**, 1237–1244.

3 Pong, A., and Bradley, J.S. (1999) Bacterial meningitis and the newborn infant. *Infect. Dis. Clin. North Am.*, **13**, 711–733, viii.

4 InVS (2008) *Réseau EPIBAC. Surveillance des infections invasives à Haemophilus influenzae, Listeria monocytogenes, Neisseria meningitidis, Streptococcus pneumoniae, Streptococcus agalactiae (B) et Streptococcus pyogenes (A) en France métropolitaine*, Institut National de Veille Sanitaire (InVs), Paris.

5 Nassif, X., Bourdoulous, S., Eugene, E., and Couraud, P.O. (2002) How do extracellular pathogens cross the blood–brain barrier? *Trends Microbiol.*, **10**, 227–232.

6 Quagliarello, V., and Scheld, W.M. (1992) Bacterial meningitis: pathogenesis, pathophysiology, and progress. *N. Engl. J. Med.*, **327**, 864–872.

7 Rubin, L.G., Zwahlen, A., and Moxon, E.R. (1985) Role of intravascular replication in the pathogenesis of experimental bacteremia due to *Haemophilus influenzae* type b. *J. Infect. Dis.*, **152**, 307–314.

8 Harrison, O.B., Robertson, B.D., Faust, S.N., Jepson, M.A., Goldin, R.D., Levin, M., and Heyderman, R.S. (2002) Analysis of pathogen–host cell interactions in purpura fulminans: expression of capsule, type IV pili, and

PorA by *Neisseria meningitidis in vivo*. *Infect. Immun.*, **70**, 5193–5201.
9 Koedel, U., Scheld, W.M., and Pfister, H.W. (2002) Pathogenesis and pathophysiology of pneumococcal meningitis. *Lancet Infect. Dis.*, **2**, 721–736.
10 Winkelstein, J.A., and Moxon, E.R. (1992) The role of complement in the host's defense against *Haemophilus influenzae*. *J. Infect. Dis.* **165** (Suppl. 1), S62–S65.
11 Austrian, R. (1981) Some observations on the pneumococcus and on the current status of pneumococcal disease and its prevention. *Rev. Infect. Dis.* **3** (Suppl.), S1–S17.
12 Moxon, E.R., and Vaughn, K.A. (1981) The type b capsular polysaccharide as a virulence determinant of *Haemophilus influenzae*: studies using clinical isolates and laboratory transformants. *J. Infect. Dis.*, **143**, 517–524.
13 Craven, D.E., Peppler, M.S., Frasch, C.E., Mocca, L.F., McGrath, P.P., and Washington, G. (1980) Adherence of isolates of *Neisseria meningitidis* from patients and carriers to human buccal epithelial cells. *J. Infect. Dis.*, **142**, 556–568.
14 Smith, A.L., Daum, R.S., Scheifele, D., Syriopoulou, V., Averil, D.R., Roberts, M.C., and Stull, T.L. (1982) Pathogenesis of *Haemophilus influenzae* meningitis, in *Haemophilus Influenzae: Epidemiology, Immunology and Prevention of the Disease* (eds S.H. Sell and P.F. Wright), Elsevier Sciences Publishing Co., Inc, New-York, pp. 89–109.
15 Drevets, D.A., Dillon, M.J., Schawang, J.S., Van Rooijen, N., Ehrchen, J., Sunderkotter, C., and Leenen, P.J. (2004) The Ly-6Chigh monocyte subpopulation transports *Listeria monocytogenes* into the brain during systemic infection of mice. *J. Immunol.*, **172**, 4418–4424.
16 Vazquez-Boland, J.A., Kuhn, M., Berche, P., Chakraborty, T., Dominguez-Bernal, G., Goebel, W., *et al.* (2001) Listeria pathogenesis and molecular virulence determinants. *Clin. Microbiol. Rev.*, **14**, 584–640.
17 Van de Beek, D., de Gans, J., Spanjaard, L., Weisfelt, M., Reitsma, J.B., and Vermeulen, M. (2004) Clinical features and prognostic factors in adults with bacterial meningitis. *N. Engl. J. Med.*, **351**, 1849–1859.
18 Pfister, H.W., Fontana, A., Tauber, M.G., Tomasz, A., and Scheld, W.M. (1994) Mechanisms of brain injury in bacterial meningitis: workshop summary. *Clin. Infect. Dis.*, **19**, 463–479.
19 Goldmann, E. (1913) Vitalfärbungen am Zentralnervensystem. Beitrag Eur Physio–Pathologie des Plexus Choroideus und der Hirnhäute. (Intravital labelling of the central nervous system. A study on the pathophysiology of the choroid plexus and the meninges.), in *Abhandlungen der königlich preußischen Akademie der Wissenschaften, Physikalisch–Mathematische Classe 1*, 1–6.
20 Ehrlich, P. (1885) *Das Sauerstoffbedürfnis des Organismus*, Eine farbenanalytische Studie, Berlin.
21 Kalimo, H., Kaste, M., and Haltia, M. (1997) Vascular disease, in *Greenfield's Neuropathology* (eds D.I. Graham and P.L. Lantos) Arnold, London, Sydney, Auckland, Oxford University Press, New York, pp. 315–396.
22 Zhang, E.T., Inman, C.B., and Weller, R.O. (1990) Interrelationships of the pia mater and the perivascular (Virchow–Robin) spaces in the human cerebrum. *J. Anat.*, **170**, 111–123.
23 Alcolado, R., Weller, R.O., Parrish, E.P., and Garrod, D. (1988) The cranial arachnoid and pia mater in man: anatomical and ultrastructural observations. *Neuropathol. Appl. Neurobiol.*, **14**, 1–17.
24 Zlokovic, B.V. (2008) The blood–brain barrier in health and chronic neurodegenerative disorders. *Neuron*, **57**, 178–201.
25 Staddon, J.M., and Rubin, L.L. (1996) Cell adhesion, cell junctions and the blood–brain barrier. *Curr. Opin. Neurobiol.*, **6**, 622–627.
26 Butt, A.M., Jones, H.C., and Abbott, N.J. (1990) Electrical resistance across the blood–brain barrier in anaesthetized rats: a developmental study. *J. Physiol.*, **429**, 47–62.
27 Crone, C., and Christensen, O. (1981) Electrical resistance of a capillary

endothelium. *J. Gen. Physiol.*, **77**, 349–371.
28 Wosik, K., Cayrol, R., Dodelet-Devillers, A., Berthelet, F., Bernard, M., Moumdjian, R., *et al.* (2007) Angiotensin II controls occludin function and is required for blood brain barrier maintenance: relevance to multiple sclerosis. *J. Neurosci.*, **27**, 9032–9042.
29 Arthur, F.E., Shivers, R.R., and Bowman, P.D. (1987) Astrocyte-mediated induction of tight junctions in brain capillary endothelium: an efficient *in vitro* model. *Brain Res.*, **433**, 155–159.
30 Abbott, N.J. (2005) Dynamics of CNS barriers: evolution, differentiation, and modulation. *Cell. Mol. Neurobiol.*, **25**, 5–23.
31 Bechmann, I., Galea, I., and Perry, V.H. (2007) What is the blood–brain barrier (not)? *Trends Immunol.*, **28**, 5–11.
32 Bechmann, I., Steiner, B., Gimsa, U., Mor, G., Wolf, S., Beyer, M., *et al.* (2002) Astrocyte-induced T cell elimination is CD95 ligand dependent. *J. Neuroimmunol.*, **132**, 60–65.
33 Kadurugamuwa, J.L., Modi, K., Coquoz, O., Rice, B., Smith, S., Contag, P.R., and Purchio, T. (2005) Reduction of astrogliosis by early treatment of pneumococcal meningitis measured by simultaneous imaging, *in vivo*, of the pathogen and host response. *Infect. Immun.*, **73**, 7836–7843.
34 Soulet, D., and Rivest, S. (2008) Microglia. *Curr. Biol.*, **18**, R506–R508.
35 Guillemin, G.J., and Brew, B.J. (2004) Microglia, macrophages, perivascular macrophages, and pericytes: a review of function and identification. *J. Leukoc. Biol.*, **75**, 388–397.
36 Thomas, W.E. (1999) Brain macrophages: on the role of pericytes and perivascular cells. *Brain Res. Brain Res. Rev.*, **31**, 42–57.
37 Broadwell, R.D., and Sofroniew, M.V. (1993) Serum proteins bypass the blood–brain fluid barriers for extracellular entry to the central nervous system. *Exp. Neurol.*, **120**, 245–263.
38 Ge, S., Song, L., and Pachter, J.S. (2005) Where is the blood–brain barrier ... really? *J. Neurosci. Res.*, **79**, 421–427.
39 Nagy, Z., Peters, H., and Huttner, I. (1984) Fracture faces of cell junctions in cerebral endothelium during normal and hyperosmotic conditions. *Lab. Invest.*, **50**, 313–322.
40 Simionescu, M., Simionescu, N., and Palade, G.E. (1975) Segmental differentiations of cell junctions in the vascular endothelium. The microvasculature. *J. Cell. Biol.*, **67**, 863–885.
41 Koh, L., Zakharov, A., and Johnston, M. (2005) Integration of the subarachnoid space and lymphatics: is it time to embrace a new concept of cerebrospinal fluid absorption? *Cerebrospinal. Fluid Res.*, **2**, 6.
42 Hutchings, M., and Weller, R.O. (1986) Anatomical relationships of the pia mater to cerebral blood vessels in man. *J. Neurosurg.*, **65**, 316–325.
43 McMenamin, P.G., Wealthall, R.J., Deverall, M., Cooper, S.J., and Griffin, B. (2003) Macrophages and dendritic cells in the rat meninges and choroid plexus: three-dimensional localisation by environmental scanning electron microscopy and confocal microscopy. *Cell Tissue Res.*, **313**, 259–269.
44 Perry, V.H., Hume, D.A., and Gordon, S. (1985) Immunohistochemical localization of macrophages and microglia in the adult and developing mouse brain. *Neuroscience*, **15**, 313–326.
45 Ahmed, A., Hickey, S.M., Ehrett, S., Trujillo, M., Brito, F., Goto, C., *et al.* (1996) Cerebrospinal fluid values in the term neonate. *Pediatr. Infect. Dis. J.*, **15**, 298–303.
46 Fishman, R.A. (1992) *Cerebrospinal Fluid in Diseases of the Nervous System*, Saunders, Philadelphia.
47 Conly, J.M., and Ronald, A.R. (1983) Cerebrospinal fluid as a diagnostic body fluid. *Am. J. Med.*, **75**, 102–108.
48 Johansson, P.A., Dziegielewska, K.M., Ek, C.J., Habgood, M.D., Liddelow, S.A., Potter, A.M., *et al.* (2006) Blood–CSF barrier function in the rat embryo. *Eur. J. Neurosci.*, **24**, 65–76.
49 Mollgard, K., and Saunders, N.R. (1986) The development of the human blood–brain and blood–CSF barriers. *Neuropathol. Appl. Neurobiol.*, **12**, 337–358.

50 Tauc, M., Vignon, X., and Bouchaud, C. (1984) Evidence for the effectiveness of the blood–CSF barrier in the fetal rat choroid plexus. A freeze-fracture and peroxidase diffusion study. *Tissue Cell*, **16**, 65–74.

51 Kim, K.S. (2003) Pathogenesis of bacterial meningitis: from bacteraemia to neuronal injury. *Nat. Rev. Neurosci.*, **4**, 376–385.

52 Syrogiannopoulos, G.A., Grivea, I.N., Anastassiou, E.D., Triga, M.G., Dimitracopoulos, G.O., and Beratis, N.G. (2001) Sterile cerebrospinal fluid pleocytosis in young infants with urinary tract infection. *Pediatr. Infect. Dis. J.*, **20**, 927–930.

53 Bergstrom, T., Larson, H., Lincoln, K., and Winberg, J. (1972) Studies of urinary tract infections in infancy and childhood. XII. Eighty consecutive patients with neonatal infection. *J. Pediatr.*, **80**, 858–866.

54 Moxon, E.R., and Ostrow, P.T. (1977) *Haemophilus influenzae* meningitis in infant rats: role of bacteremia in pathogenesis of age-dependent inflammatory responses in cerebrospinal fluid. *J. Infect. Dis.*, **135**, 303–307.

55 Doran, K.S., Engelson, E.J., Khosravi, A., Maisey, H.C., Fedtke, I., Equils, O., *et al.* (2005) Blood–brain barrier invasion by group B Streptococcus depends upon proper cell-surface anchoring of lipoteichoic acid. *J. Clin. Invest.*, **115**, 2499–2507.

56 Scolea, L., Rosales, S., Weliver, R., and Ogra, P. (1985) Mechanisms underlying the develompment of meningitis or epiglottitis in children after *Haemophilus influenzae* type b bacteraemia. *J. Infect. Dis.*, **151**, 1162–1165.

57 Zarantonelli, M.L., Lancellotti, M., Deghmane, A.E., Giorgini, D., Hong, E., Ruckly, C., *et al.* (2008) Hyperinvasive genotypes of *Neisseria meningitidis* in France. *Clin. Microbiol. Infect.*, **14**, 467–472.

58 Zarantonelli, M.L., Szatanik, M., Giorgini, D., Hong, E., Huerre, M., Guillou, F., *et al.* (2007) Transgenic mice expressing human transferrin as a model for meningococcal infection. *Infect. Immun.*, **75**, 5609–5614.

59 Weksler, B.B., Subileau, E.A., Perriere, N., Charneau, P., Holloway, K., Leveque, M., *et al.* (2005) Blood–brain barrier-specific properties of a human adult brain endothelial cell line. *FASEB J.*, **19**, 1872–1874.

60 Cundell, D.R., Gerard, C., Idanpaan-Heikkila, I., Tuomanen, E.I., and Gerard, N.P. (1996) PAf receptor anchors *Streptococcus pneumoniae* to activated human endothelial cells. *Adv. Exp. Med. Biol.*, **416**, 89–94.

61 Cundell, D.R., Gerard, N.P., Gerard, C., Idanpaan-Heikkila, I., and Tuomanen, E.I. (1995) *Streptococcus pneumoniae* anchor to activated human cells by the receptor for platelet-activating factor. *Nature*, **377**, 435–438.

62 Mairey, E., Genovesio, A., Donnadieu, E., Bernard, C., Jaubert, F., Pinard, E., *et al.* (2006) Cerebral microcirculation shear stress levels determine *Neisseria meningitidis* attachment sites along the blood–brain barrier. *J. Exp. Med.*, **203**, 1939–1950.

63 Huang, S.H., and Jong, A.Y. (2001) Cellular mechanisms of microbial proteins contributing to invasion of the blood–brain barrier. *Cell. Microbiol.*, **3**, 277–287.

64 Ring, A., Weiser, J.N., and Tuomanen, E.I. (1998) Pneumococcal trafficking across the blood–brain barrier. Molecular analysis of a novel bidirectional pathway. *J. Clin. Invest.*, **102**, 347–360.

65 Stins, M.F., Badger, J., and Sik Kim, K. (2001) Bacterial invasion and transcytosis in transfected human brain microvascular endothelial cells. *Microb. Pathog.*, **30**, 19–28.

66 Prasadarao, N.V., Wass, C.A., Stins, M.F., and Kim, K.S. (1999) Outer membrane protein A-promoted actin condensation of brain microvascular endothelial cells is required for *Escherichia coli* invasion. *Infect. Immun.*, **67**, 5775–5783.

67 Stins, M.F., Nemani, P.V., Wass, C., and Kim, K.S. (1999) *Escherichia coli* binding to and invasion of brain microvascular endothelial cells derived from humans and rats of different ages. *Infect. Immun.*, **67**, 5522–5525.

68 Nizet, V., Kim, K.S., Stins, M., Jonas, M., Chi, E.Y., Nguyen, D., and Rubens, C.E. (1997) Invasion of brain microvascular endothelial cells by group B streptococci. *Infect. Immun.*, **65**, 5074–5081.

69 Virji, M., Kayhty, H., Ferguson, D.J., Alexandrescu, C., and Moxon, E.R. (1992) Interactions of *Haemophilus influenzae* with human endothelial cells *in vitro*. *J. Infect. Dis.* **165** (Suppl. 1): S115-S116.

70 Virji, M., Kayhty, H., Ferguson, D.J., Alexandrescu, C., and Moxon, E.R. (1991) Interactions of *Haemophilus influenzae* with cultured human endothelial cells. *Microb. Pathog.*, **10**, 231–245.

71 Nikulin, J., Panzner, U., Frosch, M., and Schubert-Unkmeir, A. (2006) Intracellular survival and replication of *Neisseria meningitidis* in human brain microvascular endothelial cells. *Int. J. Med. Microbiol.*, **296**, 553–558.

72 Tuomanen, E. (1996) Entry of pathogens into the central nervous system. *FEMS Microbiol. Rev.*, **18**, 289–299.

73 Smith, A.L. (1987) Pathogenesis of *Haemophilus influenzae* meningitis. *Pediatr. Infect. Dis. J.*, **6**, 783–786.

74 Dietzman, D.E., Fischer, G.W., and Schoenknecht, F.D. (1974) Neonatal *Escherichia coli* septicemia – bacterial counts in blood. *J. Pediatr.*, **85**, 128–130.

75 Mogensen, T.H., Paludan, S.R., Kilian, M., and Ostergaard, L. (2006) Live *Streptococcus pneumoniae, Haemophilus influenzae*, and *Neisseria meningitidis* activate the inflammatory response through Toll-like receptors 2, 4, and 9 in species-specific patterns. *J. Leukoc. Biol.*, **80**, 267–277.

76 Johansson, L., Rytkonen, A., Wan, H., Bergman, P., Plant, L., Agerberth, B., *et al.* (2005) Human-like immune responses in CD46 transgenic mice. *J. Immunol.*, **175**, 433–440.

77 Nassif, X., Mathison, J.C., Wolfson, E., Koziol, J.A., Ulevitch, R.J., and So, M. (1992) Tumour necrosis factor alpha antibody protects against lethal meningococcaemia. *Mol. Microbiol.*, **6**, 591–597.

78 Thijs, L.G., and Hack, C.E. (1995) Time course of cytokine levels in sepsis. *Intensive Care Med.* **21** (Suppl. 2), S258–S263.

79 Davies, D.C. (2002) Blood–brain barrier breakdown in septic encephalopathy and brain tumours. *J. Anat.*, **200**, 639–646.

80 Papadopoulos, M.C., Davies, D.C., Moss, R.F., Tighe, D., and Bennett, E.D. (2000) Pathophysiology of septic encephalopathy: a review. *Crit. Care Med.*, **28**, 3019–3024.

81 Miller, F., Fenart, L., Landry, V., Coisne, C., Cecchelli, R., Dehouck, M.P., and Buee-Scherrer, V. (2005) The MAP kinase pathway mediates transcytosis induced by TNF-alpha in an *in vitro* blood–brain barrier model. *Eur. J. Neurosci.*, **22**, 835–844.

82 Alexander, J.J., Jacob, A., Cunningham, P., Hensley, L., and Quigg, R.J. (2008) TNF is a key mediator of septic encephalopathy acting through its receptor, TNF receptor-1. *Neurochem. Int.*, **52**, 447–456.

83 Freyer, D., Manz, R., Ziegenhorn, A., Weih, M., Angstwurm, K., Docke, W.D., *et al.* (1999) Cerebral endothelial cells release TNF-alpha after stimulation with cell walls of *Streptococcus pneumoniae* and regulate inducible nitric oxide synthase and ICAM-1 expression via autocrine loops. *J. Immunol.*, **163**, 4308–4314.

84 Abbott, N.J., Ronnback, L., and Hansson, E. (2006) Astrocyte–endothelial interactions at the blood–brain barrier. *Nat. Rev. Neurosci.*, **7**, 41–53.

85 Abbott, N. (2002) Astrocyte–endothelial interactions and blood–brain barrier permeability. *J. Anat.*, **200**, 527.

86 Ge, S., Song, L., Serwanski, D.R., Kuziel, W.A., and Pachter, J.S. (2008) Transcellular transport of CCL2 across brain microvascular endothelial cells. *J. Neurochem.*, **104**, 1219–1232.

87 Mordelet, E., Davies, H.A., Hillyer, P., Romero, I.A., and Male, D. (2007) Chemokine transport across human vascular endothelial cells. *Endothelium*, **14**, 7–15.

88 Pan, W., Kastin, A.J., Daniel, J., Yu, C., Baryshnikova, L.M., and von Bartheld,

C.S. (2007) TNFα trafficking in cerebral vascular endothelial cells. *J. Neuroimmunol.*, **185**, 47–56.

89 Romanovsky, A.A., Almeida, M.C., Aronoff, D.M., Ivanov, A.I., Konsman, J.P., Steiner, A.A., and Turek, V.F. (2005) Fever and hypothermia in systemic inflammation: recent discoveries and revisions. *Front. Biosci.*, **10**, 2193–2216.

90 Kielian, T. (2006) Toll-like receptors in central nervous system glial inflammation and homeostasis. *J. Neurosci. Res.*, **83**, 711–730.

91 Nimmerjahn, A., Kirchhoff, F., and Helmchen, F. (2005) Resting microglial cells are highly dynamic surveillants of brain parenchyma *in vivo*. *Science*, **308**, 1314–1318.

92 Aloisi, F. (2001) Immune function of microglia. *Glia*, **36**, 165–179.

93 Dong, Y., and Benveniste, E.N. (2001) Immune function of astrocytes. *Glia*, **36**, 180–190.

94 Persidsky, Y., Ghorpade, A., Rasmussen, J., Limoges, J., Liu, X.J., Stins, M., *et al.* (1999) Microglial and astrocyte chemokines regulate monocyte migration through the blood–brain barrier in human immunodeficiency virus-1 encephalitis. *Am. J. Pathol.*, **155**, 1599–1611.

95 Wang, T., Town, T., Alexopoulou, L., Anderson, J.F., Fikrig, E., and Flavell, R.A. (2004) Toll-like receptor 3 mediates West Nile virus entry into the brain causing lethal encephalitis. *Nat. Med.*, **10**, 1366–1373.

96 Parry, S., Zhang, J., Koi, H., Arechavaleta-Velasco, F., and Elovitz, M.A. (2006) Transcytosis of Human immunodeficiency virus 1 across the placenta is enhanced by treatment with tumour necrosis factor alpha. *J. Gen. Virol.*, **87**, 2269–2278.

97 Fiala, M., Looney, D.J., Stins, M., Way, D.D., Zhang, L., Gan, X., *et al.* (1997) TNF-alpha opens a paracellular route for HIV-1 invasion across the blood–brain barrier. *Mol. Med.*, **3**, 553–564.

98 Stoll, B.J. (1997) The global impact of neonatal infection. *Clin. Perinatol.*, **24**, 1–21.

99 de Louvois, J. (1994) Acute bacterial meningitis in the newborn. *J. Antimicrob. Chemother.* **34** (Suppl. A), 61–73.

100 Fortnum, H.M., and Davis, A.C. (1993) Epidemiology of bacterial meningitis. *Arch. Dis. Child.*, **68**, 763–767.

101 Hristeva, L., Booy, R., Bowler, I., and Wilkinson, A.R. (1993) Prospective surveillance of neonatal meningitis. *Arch. Dis. Child.*, **69**, 14–18.

102 de Louvois, J., Blackbourn, J., Hurley, R., and Harvey, D. (1991) Infantile meningitis in England and Wales: a two year study. *Arch. Dis. Child.*, **66**, 603–607.

103 Overall, J.C., Jr. (1970) Neonatal bacterial meningitis. Analysis of predisposing factors and outcome compared with matched control subjects. *J. Pediatr.*, **76**, 499–511.

104 Houdouin, V., Bonacorsi, S., Bidet, P., Blanco, J., De La Rocque, F., Cohen, R., *et al.* (2008) Association between mortality of *Escherichia coli* meningitis in young infants and non-virulent clonal groups of strains. *Clin. Microbiol. Infect.*, **14**, 685–690.

105 May, M., Daley, A.J., Donath, S., and Isaacs, D. (2005) Early onset neonatal meningitis in Australia and New Zealand, 1992–2002. *Arch. Dis. Child. Fetal Neonatal Ed.*, **90**, F324–F327.

106 Stoll, B.J., Hansen, N.I., Higgins, R.D., Fanaroff, A.A., Duara, S., Goldberg, R., *et al.* (2005) Very low birth weight preterm infants with early onset neonatal sepsis: the predominance of gram-negative infections continues in the National Institute of Child Health and Human Development Neonatal Research Network, 2002–2003. *Pediatr. Infect. Dis. J.*, **24**, 635–639.

107 Unhanand, M., Mustafa, M.M., McCracken, G.H., Jr., and Nelson, J.D. (1993) Gram-negative enteric bacillary meningitis: a twenty-one-year experience. *J. Pediatr.*, **122**, 15–21.

108 Pluschke, G., Mercer, A., Kusecek, B., Pohl, A., and Achtman, M. (1983) Induction of bacteremia in newborn rats by *Escherichia coli* K1 is correlated with only certain O (lipopolysaccharide)

antigen types. *Infect. Immun.*, **39**, 599–608.

109 Glode, M.P., Sutton, A., Robbins, J.B., McCracken, G.H., Gotschlich, E.C., Kaijser, B., and Hanson, L.A. 1977b. Neonatal meningitis due of *Escherichia coli* K1. *J. Infect. Dis.* **136** (Suppl.), S93–S97.

110 Kim, K.S., Itabashi, H., Gemski, P., Sadoff, J., Warren, R.L., and Cross, A.S. (1992) The K1 capsule is the critical determinant in the development of *Escherichia coli* meningitis in the rat. *J. Clin. Invest.*, **90**, 897–905.

111 Houdouin, V., Bonacorsi, S., Brahimi, N., Clermont, O., Nassif, X., and Bingen, E. (2002) A uropathogenicity island contributes to the pathogenicity of *Escherichia coli* strains that cause neonatal meningitis. *Infect. Immun.*, **70**, 5865–5869.

112 Selander, R.K., Caugant, D.A., and Whittam, T.S. (1987) Genetic structure and variation in natural populations of *Escherichia coli*, in *Escherichia coli and Salmonella typhimurium: Cellular and Molecular Biology* (eds F.C. Neidhardt, J.L. Ingraham, K.B. Low, B. Magasanik, M. Schaechter, and H.E. Umbarger) American Society for Microbiology, Washington D.C., pp. 1625–1628.

113 Achtman, M., Mercer, A., Kusecek, B., Pohl, A., Heuzenroeder, M., Aaronson, W., *et al.* (1983) Six widespread bacterial clones among *Escherichia coli* K1 isolates. *Infect. Immun.*, **39**, 315–335.

114 Sarff, L.D., McCracken, G.H., Schiffer, M.S., Glode, M.P., Robbins, J.B., Orskov, I., and Orskov, F. (1975) Epidemiology of *Escherichia coli* K1 in healthy and diseased newborns. *Lancet*, **1**, 1099–1104.

115 Bonacorsi, S., Clermont, O., Houdouin, V., Cordevant, C., Brahimi, N., Marecat, A., *et al.* (2003) Molecular analysis and experimental virulence of French and North American *Escherichia coli* neonatal meningitis isolates: identification of a new virulent clone. *J. Infect. Dis.*, **187**, 1895–1906.

116 Mulder, C.J., van Alphen, L., and Zanen, H.C. (1984) Neonatal meningitis caused by *Escherichia coli* in The Netherlands. *J. Infect. Dis.*, **150**, 935–940.

117 Bidet, P., Mahjoub-Messai, F., Blanco, J., Blanco, J., Dehem, M., Aujard, Y., *et al.* (2007) Combined multilocus sequence typing and O serogrouping distinguishes *Escherichia coli* subtypes associated with infant urosepsis and/or meningitis. *J. Infect. Dis.*, **196**, 297–303.

118 Huang, S.H., Wass, C., Fu, Q., Prasadarao, N.V., Stins, M., and Kim, K.S. (1995) *Escherichia coli* invasion of brain microvascular endothelial cells *in vitro* and *in vivo*: molecular cloning and characterization of invasion gene ibe10. *Infect. Immun.*, **63**, 4470–4475.

119 Bortolussi, R., Ferrieri, P., and Wannamaker, L.W. (1978) Dynamics of *Escherichia coli* infection and meningitis in infant rats. *Infect. Immun.*, **22**, 480–485.

120 Glode, M.P., Sutton, A., Moxon, E.R., and Robbins, J.B. (1977) Pathogenesis of neonatal *Escherichia coli* meningitis: induction of bacteremia and meningitis in infant rats fed *E. coli* K1. *Infect. Immun.*, **16**, 75–80.

121 Bloch, C.A., Huang, S.H., Rode, C.K., and Kim, K.S. (1996) Mapping of noninvasion TnphoA mutations on the *Escherichia coli* O18:K1:H7 chromosome. *FEMS Microbiol. Lett.*, **144**, 171–176.

122 Bonacorsi, S.P., Clermont, O., Tinsley, C., Le Gall, I., Beaudoin, J.C., Elion, J., *et al.* (2000) Identification of regions of the *Escherichia coli* chromosome specific for neonatal meningitis-associated strains. *Infect. Immun.*, **68**, 2096–2101.

123 Gonzalez, M.D., Lichtensteiger, C.A., and Vimr, E.R. (2001) Adaptation of signature-tagged mutagenesis to *Escherichia coli* K1 and the infant-rat model of invasive disease. *FEMS. Microbiol. Lett.*, **198**, 125–128.

124 Badger, J.L., Wass, C.A., Weissman, S.J., and Kim, K.S. (2000) Application of signature-tagged mutagenesis for identification of *Escherichia coli* K1 genes that contribute to invasion of human brain microvascular endothelial cells. *Infect. Immun.*, **68**, 5056–5061.

125 Martindale, J., Stroud, D., Moxon, E.R., and Tang, C.M. (2000) Genetic analysis of *Escherichia coli* K1 gastrointestinal colonization. *Mol. Microbiol.*, **37**, 1293–1305.

126 Badger, J.L., Wass, C.A., and Kim, K.S. (2000) Identification of *Escherichia coli* K1 genes contributing to human brain microvascular endothelial cell invasion by differential fluorescence induction. *Mol. Microbiol.*, **36**, 174–182.

127 Xie, Y., Kim, K.J., and Kim, K.S. (2004) Current concepts on *Escherichia coli* K1 translocation of the blood–brain barrier. *FEMS Immunol. Med. Microbiol.*, **42**, 271–279.

128 Plainvert, C., Bidet, P., Peigne, C., Barbe, V., Medigue, C., Denamur, E., *et al.* (2007) A new O-antigen gene cluster has a key role in the virulence of the *Escherichia coli* meningitis clone O45:K1:H7. *J. Bacteriol.*, **189**, 8528–8536.

129 Xie, Y., Kolisnychenko, V., Paul-Satyaseela, M., Elliott, S., Parthasarathy, G., Yao, Y., *et al.* (2006) Identification and characterization of *Escherichia coli* RS218-derived islands in the pathogenesis of *E. coli* meningitis. *J. Infect. Dis.*, **194**, 358–364.

130 Ewers, C., Li, G., Wilking, H., Kiessling, S., Alt, K., Antao, E.M., *et al.* (2007) Avian pathogenic, uropathogenic, and newborn meningitis-causing *Escherichia coli*: how closely related are they? *Int. J. Med. Microbiol.*, **297**, 163–176.

131 Silver, R.P., Aaronson, W., and Vann, W.F. (1988) The K1 capsular polysaccharide of *Escherichia coli*. *Rev. Infect. Dis.*, **10** (Suppl. 2), S282–S286.

132 Rick, P.D., and Silver, R.P. (1996) Enterobacterial common antigen and capsular polysaccharides, in *Escherichia coli and Salmonella Typhimurium: Cellular and Molecular Biology* (eds F.C. Neidhardt, J.L. Ingraham, K.B. Low, B. Magasanik, M. Schaechter, and H.E. Umbarger), American Society for Microbiology, Washington D.C., pp. 104–122.

133 Pluschke, G., and Achtman, M. (1984) Degree of antibody-independent activation of the classical complement pathway by K1 *Escherichia coli* differs with O antigen type and correlates with virulence of meningitis in newborns. *Infect. Immun.*, **43**, 684–692.

134 Negre, V.L., Bonacorsi, S., Schubert, S., Bidet, P., Nassif, X., and Bingen, E. (2004) The siderophore receptor IroN, but not the high-pathogenicity island or the hemin receptor ChuA, contributes to the bacteremic step of *Escherichia coli* neonatal meningitis. *Infect. Immun.*, **72**, 1216–1220.

135 Swenson, D.L., Bukanov, N.O., Berg, D.E., and Welch, R.A. (1996) Two pathogenicity islands in uropathogenic *Escherichia coli* J96: cosmid cloning and sample sequencing. *Infect. Immun.*, **64**, 3736–3743.

136 Teng, C.H., Cai, M., Shin, S., Xie, Y., Kim, K.J., Khan, N.A., *et al.* (2005) *Escherichia coli* K1 RS218 interacts with human brain microvascular endothelial cells via type 1 fimbria bacteria in the fimbriated state. *Infect. Immun.*, **73**, 2923–2931.

137 Prasadarao, N.V., Wass, C.A., Weiser, J.N., Stins, M.F., Huang, S.H., and Kim, K.S. (1996) Outer membrane protein A of *Escherichia coli* contributes to invasion of brain microvascular endothelial cells. *Infect. Immun.*, **64**, 146–153.

138 Kim, K.S. (2002) Strategy of *Escherichia coli* for crossing the blood–brain barrier. *J. Infect. Dis.* **186** (Suppl. 2), S220–S224.

139 Khan, N.A., Wang, Y., Kim, K.J., Chung, J.W., Wass, C.A., and Kim, K.S. (2002) Cytotoxic necrotizing factor-1 contributes to *Escherichia coli* K1 invasion of the central nervous system. *J. Biol. Chem.*, **277**, 15607–15612.

140 Hoffman, J.A., Wass, C., Stins, M.F., and Kim, K.S. (1999) The capsule supports survival but not traversal of *Escherichia coli* K1 across the blood–brain barrier. *Infect. Immun.*, **67**, 3566–3570.

141 Wolach, B., Dolfin, T., Regev, R., Gilboa, S., and Schlesinger, M. (1997) The development of the complement system after 28 weeks' gestation. *Acta Paediatr.*, **86**, 523–527.

142 Lassiter, H.A., Walz, B.M., Wilson, J.L., Jung, E., Calisi, C.R., Goldsmith, L.J., *et al.* (1997) The administration of

complement component C9 enhances the survival of neonatal rats with *Escherichia coli* sepsis. *Pediatr. Res.*, **42**, 128–136.

143 Levy, O., Martin, S., Eichenwald, E., Ganz, T., Valore, E., Carroll, S.F., *et al.* (1999) Impaired innate immunity in the newborn: newborn neutrophils are deficient in bactericidal/permeability-increasing protein. *Pediatrics*, **104**, 1327–1333.

144 Sadeghi, K., Berger, A., Langgartner, M., Prusa, A.R., Hayde, M., Herkner, K., *et al.* (2007) Immaturity of infection control in preterm and term newborns is associated with impaired toll-like receptor signaling. *J. Infect. Dis.*, **195**, 296–302.

145 Edwards, M.S., and Baker, C.J. (2005) Group B streptococcal infections, in *Infectious Diseases of the Fetus and Newborn Infant* (eds J.O. Klein and J.S. Remington), Saunders, pp. 1091–1156.

146 Jones, N., Oliver, K.A., Barry, J., Harding, R.M., Bisharat, N., Spratt, B.G., *et al.* (2006) Enhanced invasiveness of bovine-derived neonatal sequence type 17 group B streptococcus is independent of capsular serotype. *Clin. Infect. Dis.*, **42**, 915–924.

147 Lamy, M.C., Dramsi, S., Billoet, A., Reglier-Poupet, H., Tazi, A., Raymond, J., *et al.* (2006) Rapid detection of the 'highly virulent' group B Streptococcus ST-17 clone. *Microbes. Infect.*, **8**, 1714–1722.

148 Lin, F.Y., Whiting, A., Adderson, E., Takahashi, S., Dunn, D.M., Weiss, R., *et al.* (2006) Phylogenetic lineages of invasive and colonizing strains of serotype III group B Streptococci from neonates: a multicenter prospective study. *J. Clin. Microbiol.*, **44**, 1257–1261.

149 Fluegge, K., Supper, S., Siedler, A., and Berner, R. (2005) Serotype distribution of invasive group B streptococcal isolates in infants: results from a nationwide active laboratory surveillance study over 2 years in Germany. *Clin. Infect. Dis.*, **40**, 760–763.

150 Jones, N., Bohnsack, J.F., Takahashi, S., Oliver, K.A., Chan, M.S., Kunst, F., *et al.* (2003) Multilocus sequence typing system for group B streptococcus. *J. Clin. Microbiol.*, **41**, 2530–2536.

151 Poyart, C., Reglier-Poupet, H., Tazi, A., Billoet, A., Dmytruk, N., Bidet, P., *et al.* (2008) Invasive group B streptococcal infections in infants: a 18-month French Nationwide Survey (2006–2007). *Emerg. Infect. Dis.*, **14**, 1647–1649.

152 Ferrieri, P., Burke, B., and Nelson, J. (1980) Production of bacteremia and meningitis in infant rats with group B streptococcal serotypes. *Infect. Immun.*, **27**, 1023–1032.

153 Doran, K.S., and Nizet, V. (2004) Molecular pathogenesis of neonatal group B streptococcal infection: no longer in its infancy. *Mol. Microbiol.*, **54**, 23–31.

154 Angata, T., and Varki, A. (2002) Chemical diversity in the sialic acids and related alpha-keto acids: an evolutionary perspective. *Chem. Rev.*, **102**, 439–469.

155 Wessels, M.R., Rubens, C.E., Benedi, V.J., and Kasper, D.L. (1989) Definition of a bacterial virulence factor: sialylation of the group B streptococcal capsule. *Proc. Natl. Acad. Sci. USA*, **86**, 8983–8987.

156 Pritzlaff, C.A., Chang, J.C., Kuo, S.P., Tamura, G.S., Rubens, C.E., and Nizet, V. (2001) Genetic basis for the beta-haemolytic/cytolytic activity of group B Streptococcus. *Mol. Microbiol.*, **39**, 236–247.

157 Rosa-Fraile, M., Rodriguez-Granger, J., Haidour-Benamin, A., Cuerva, J.M., and Sampedro, A. (2006) Granadaene: proposed structure of the group B Streptococcus polyenic pigment. *Appl. Environ. Microbiol.*, **72**, 6367–6370.

158 Doran, K.S., Liu, G.Y., and Nizet, V. (2003) Group B streptococcal beta-hemolysin/cytolysin activates neutrophil signaling pathways in brain endothelium and contributes to development of meningitis. *J. Clin. Invest.*, **112**, 736–744.

159 Tenenbaum, T., Spellerberg, B., Adam, R., Vogel, M., Kim, K.S., and Schroten, H. (2007) *Streptococcus agalactiae* invasion of human brain microvascular endothelial cells is promoted by the laminin-binding protein Lmb. *Microbes Infect.*, **9**, 714–720.

160 Tenenbaum, T., Bloier, C., Adam, R., Reinscheid, D.J., and Schroten, H.

(2005) Adherence to and invasion of human brain microvascular endothelial cells are promoted by fibrinogen-binding protein FbsA of *Streptococcus agalactiae*. *Infect. Immun.*, **73**, 4404–4409.

161 Dramsi, S., Caliot, E., Bonne, I., Guadagnini, S., Prevost, M.C., Kojadinovic, M., *et al.* (2006) Assembly and role of pili in group B streptococci. *Mol. Microbiol.*, **60**, 1401–1413.

162 Rosini, R., Rinaudo, C.D., Soriani, M., Lauer, P., Mora, M., Maione, D., *et al.* (2006) Identification of novel genomic islands coding for antigenic pilus-like structures in *Streptococcus agalactiae*. *Mol. Microbiol.*, **61**, 126–141.

163 Telford, J.L., Barocchi, M.A., Margarit, I., Rappuoli, R., and Grandi, G. (2006) Pili in gram-positive pathogens. *Nat. Rev. Microbiol.*, **4**, 509–519.

164 Maisey, H.C., Hensler, M., Nizet, V., and Doran, K.S. (2007) Group B streptococcal pilus proteins contribute to adherence to and invasion of brain microvascular endothelial cells. *J. Bacteriol.*, **189**, 1464–1467.

165 Bille, E., Zahar, J.R., Perrin, A., Morelle, S., Kriz, P., Jolley, K.A., *et al.* (2005) A chromosomally integrated bacteriophage in invasive meningococci. *J. Exp. Med.*, **201**, 1905–1913.

166 Seib, K.L., Serruto, D., Oriente, F., Delany, I., Adu-Bobie, J., Veggi, D., *et al.* (2008) Factor H-binding protein is important for meningococcal survival in human whole blood and serum, and in the presence of the antimicrobial peptide LL-37. *Infect. Immun.*

167 Beernink, P.T., Leipus, A., and Granoff, D.M. (2006) Rapid genetic grouping of factor H-binding protein (genome-derived neisserial antigen 1870), a promising group B meningococcal vaccine candidate. *Clin. Vaccine Immunol.*, **13**, 758–763.

168 Guarner, J., Greer, P.W., Whitney, A., Shieh, W.J., Fischer, M., White, E.H., *et al.* (2004) Pathogenesis and diagnosis of human meningococcal disease using immunohistochemical and PCR assays. *Am. J. Clin. Pathol.*, **122**, 754–764.

169 Lory, S., and Strom, M.S. (1997) Structure–function relationship of type-IV prepilin peptidase of *Pseudomonas aeruginosa* – a review. *Gene*, **192**, 117–121.

170 Helaine, S., Dyer, D.H., Nassif, X., Pelicic, V., and Forest, K.T. (2007) 3D structure/function analysis of PilX reveals how minor pilins can modulate the virulence properties of type IV pili. *Proc. Natl. Acad. Sci. USA*, **104**, 15888–15893.

171 Helaine, S., Carbonnelle, E., Prouvensier, L., Beretti, J.L., Nassif, X., and Pelicic, V. (2005) PilX, a pilus-associated protein essential for bacterial aggregation, is a key to pilus-facilitated attachment of *Neisseria meningitidis* to human cells. *Mol. Microbiol.*, **55**, 65–77.

172 Kallstrom, H., Liszewski, M.K., Atkinson, J.P., and Jonsson, A.B. (1997) Membrane cofactor protein (MCP or CD46) is a cellular pilus receptor for pathogenic Neisseria. *Mol. Microbiol.*, **25**, 639–647.

173 Johansson, L., Rytkonen, A., Bergman, P., Albiger, B., Kallstrom, H., Hokfelt, T., *et al.* (2003) CD46 in meningococcal disease. *Science*, **301**, 373–375.

174 Kirchner, M., Heuer, D., and Meyer, T.F. (2005) CD46-independent binding of neisserial type IV pili and the major pilus adhesin, PilC, to human epithelial cells. *Infect. Immun.*, **73**, 3072–3082.

175 Pujol, C., Eugene, E., de Saint Martin, L., and Nassif, X. (1997) Interaction of *Neisseria meningitidis* with a polarized monolayer of epithelial cells. *Infect. Immun.*, **65**, 4836–4842.

176 Merz, A.J., Rifenbery, D.B., Arvidson, C.G., and So, M. (1996) Traversal of a polarized epithelium by pathogenic Neisseriae: facilitation by type IV pili and maintenance of epithelial barrier function. *Mol. Med.*, **2**, 745–754.

177 Eugene, E., Hoffmann, I., Pujol, C., Couraud, P.O., Bourdoulous, S., and Nassif, X. (2002) Microvilli-like structures are associated with the internalization of virulent capsulated *Neisseria meningitidis* into vascular endothelial cells. *J. Cell Sci.*, **115**, 1231–1241.

178 Merz, A.J., Enns, C.A., and So, M. (1999) Type IV pili of pathogenic Neisseriae elicit cortical plaque

formation in epithelial cells. *Mol. Microbiol.*, **32**, 1316–1332.

179 Merz, A.J., and So, M. (1997) Attachment of piliated, Opa- and Opc- gonococci and meningococci to epithelial cells elicits cortical actin rearrangements and clustering of tyrosine-phosphorylated proteins. *Infect. Immun.*, **65**, 4341–4349.

180 Lambotin, M., Hoffmann, I., Laran-Chich, M.P., Nassif, X., Couraud, P.O., and Bourdoulous, S. (2005) Invasion of endothelial cells by *Neisseria meningitidis* requires cortactin recruitment by a phosphoinositide-3-kinase/Rac1 signalling pathway triggered by the lipo-oligosaccharide. *J. Cell Sci.*, **118**, 3805–3816.

181 Hoffmann, I., Eugene, E., Nassif, X., Couraud, P.O., and Bourdoulous, S. (2001) Activation of ErbB2 receptor tyrosine kinase supports invasion of endothelial cells by *Neisseria meningitidis*. *J. Cell Biol.*, **155**, 133–143.

182 Merz, A.J., So, M., and Sheetz, M.P. (2000) Pilus retraction powers bacterial twitching motility. *Nature*, **407**, 98–102.

183 Barreiro, O., Yanez-Mo, M., Sala-Valdes, M., Gutierrez-Lopez, M.D., Ovalle, S., Higginbottom, A., *et al.* (2005) Endothelial tetraspanin microdomains regulate leukocyte firm adhesion during extravasation. *Blood*, **105**, 2852–2861.

184 Carman, C.V., and Springer, T.A. (2004) A transmigratory cup in leukocyte diapedesis both through individual vascular endothelial cells and between them. *J. Cell Biol.*, **167**, 377–388.

185 Shaw, S.K., Ma, S., Kim, M.B., Rao, R.M., Hartman, C.U., Froio, R.M., *et al.* (2004) Coordinated redistribution of leukocyte LFA-1 and endothelial cell ICAM-1 accompany neutrophil transmigration. *J. Exp. Med.*, **200**, 1571–1580.

186 Doulet, N., Donnadieu, E., Laran-Chich, M.P., Niedergang, F., Nassif, X., Couraud, P.O., and Bourdoulous, S. (2006) Neisseria *meningitidis* infection of human endothelial cells interferes with leukocyte transmigration by preventing the formation of endothelial docking structures. *J. Cell. Biol.*, **173**, 627–637.

6
Two Important Bacterial Pathogens causing Community Acquired Pneumonia: *Streptococcus pneumoniae* and *Legionella pneumophila*

Birgitta Henriques-Normark and Carmen Buchrieser

6.1
General Background

Acute lower respiratory tract infections (ARI) are the leading cause of morbidity and mortality worldwide among infectious diseases. They remain one of the main causes of childhood mortality in the world with an estimated 1 to 1.5 million deaths among children under the age of 5 years, of which the predominant number of cases are due to pneumonia. Community acquired pneumonia, CAP, is defined as an infection of the pulmonary parenchyma associated with at least some symptoms/signs of acute infection as well as the presence of acute infiltrates on chest radiographs in patients that have not been hospitalized 2 weeks prior to the onset of symptoms [1]. It is estimated that 5–6 million people acquire CAP every year in the USA and about 1 million are hospitalized. Many studies have been carried out to study the etiology of CAP and most studies show that the major causative agent is the bacterium *Streptococcus pneumoniae* or pneumococci. Other so-called 'typical bacteria' that cause CAP are *Haemophilus influenzae* and *Moraxella catarrhalis*. Bacteria including *Mycoplasma pneumoniae*, *Chlamydia* spp, and *Legionella* spp can also be the causative agents of CAP and are traditionally known as atypical bacteria because they differ both clinically and microbiologically from the typical species. *Streptococcus pneumoniae* is also the most common cause of CAP in hospitalized patients and in fatal cases, whereas *Legionella* is a common cause of severe CAP requiring admission to intensive care units [2]. However, diagnosis of CAP is not trivial and even though several different methods are in use only a proportion of CAP cases are etiologically diagnosed. Typical symptoms of CAP include for example productive cough, dyspnoea, pleuritic chest pains, fever, tachypnea and tachycardia. However, elderly patients in particular, may not present with all these symptoms and may also report non-respiratory symptoms such as headache, fatigue etc. as the main clinical manifestations.

The type of groups at risk for acquiring CAP depends on the etiological agent to some extent. In the case of pneumococcal pneumonia, small children, especially those below the age of 2 years, and the elderly as well as immuno-compromised

Bacterial Virulence: Basic Principles, Models and Global Approaches. Edited by Philippe Sansonetti
© 2010 WILEY-VCH Verlag GmbH & Co. KGaA, Weinheim
ISBN: 978-3-527-32326-5

individuals such as HIV patients, are more prone to infection. Also, alcoholics and smokers are more susceptible as well as splenectomized individuals. Pneumococci are common colonizers of the upper respiratory tract of small children and from this ecological niche they may spread to susceptible individuals such as elderly grand parents and cause pneumonia. The elderly have an increased risk for CAP since many have co-morbidities such as heart and lung diseases, but also the mucociliary clearance and the cough reflex may be impaired. In 20–30% of cases with pneumonia the bacteria are also found in the blood causing septicemia and they may also bypass the blood–brain barrier to cause meningitis. These invasive diseases have a higher mortality rate than pneumonia cases and it is estimated that in the USA 5% of patients die from pneumococcal pneumonia, 20% from septicemia and as high as 30% from meningitis [3]. Up to 30% of survivors of meningitis may also suffer neurological sequelae, such as hearing loss, memory impairment etc. Pneumococcal diseases show a seasonal variation, with more infections occurring during winter seasons. In addition, there is an association with viral diseases and during the influenza season in particular the number of cases of pneumococcal pneumonia and invasive disease increases (also see below).

Legionella is an opportunistic pathogen to which immuno-suppressed and elderly patients are the most vulnerable. *Legionella* consist of one genus with 50 species and more than 70 serogroups. About 50% of these have at least once been isolated from human disease whilst the others are found only in the environment. Legionnaires' disease is caused by *Legionella pneumophila* and was first recognized in 1976 in Philadelphia at an American Legionnaires convention. Symptoms included fever, disorientation and lung damage, and in some cases other organs such as the gastrointestinal tract, central nervous system and liver and/or kidneys were affected. The bacteria were found to be spread to humans in aerosols emanating from air conditioning systems. *Legionella* bacteria are able to survive in water systems and amoebae, thus susceptible individuals may be infected from showers and other water supplies in institutions such as hospitals. However, these bacteria are not passed from person to person. *Legionella* may cause outbreaks of severe pneumonia, and most epidemics appear during late summer/autumn with a high mortality rate of about 15–20%.

Treatment of both pneumococcal infections and *Legionella* pneumonia is antibiotics. Penicillin has been the drug of choice for the treatment of pneumococcal infections for many years but due to the emergence of resistance to the drug in many parts of the world, over 50% in some areas, other groups of antibiotics have been used. To date resistance has been found to most antibiotics, the only exception being vancomycin. *Legionella* is treated with antibiotics other than penicillin, for example macrolides or tetracyclines. Definitive diagnosis of *Legionella* infection is a major problem since this bacterium is fastidious and requires complex nutritional media for growth. Hence, *Legionella* must be suspected before the clinician can make the diagnosis. Therefore, it is probable that not all *Legionella* cases are identified.

In contrast to pneumococci, *Legionella* are Gram-negative intracellular rods that can survive and multiply inside macrophages. However, it remains to be seen

whether or not pneumococci are also capable of surviving for a limited period of time within phagocytes such as macrophages and dendritic cells. *Legionella* enters the phagocytes via the C3b receptor (CR3) and inhibits the fusion of phagosome and lysosome and kills the macrophages by means of proteases and other secreted proteins. This leads to an inflammatory response and micro-abscesses resulting in impaired lung function. In typical pneumococcal pneumonia the bacteria first colonize the upper airways and nasopharynx, and are then aspirated into the lower airways and multiply in the alveolar spaces leading to local hyperemia, edema and the recruitment of neutrophils (congestion). Capillaries and alveoli then become filled with bacteria and red blood cells (red hepatization) and then invaded by first neutrophils and later by macrophages. The bacteria are then phagocytosed leading to the infected part of the lung becoming gray-white in color (gray hepatization). After this the resolution phase occurs and the lungs usually heal without any scarring. The infiltrates observed by chest radiography are usually localized to one or two lobes.

Pneumococci are Gram-positive cocci which are usually seen as diplococci or small chains. They can be divided into serogroups and serotypes depending on the differences in their capsular polysaccharides. So far 91 different capsular serotypes have been distinguished. The serotype distribution among invasive cases differs depending on time course of the infection and geographical area studied under study. The capsule is a major virulence factor (described below) and the current pneumococcal vaccines are based either on the capsular polysaccharides alone or in combination with a protein in conjugated vaccines. For several decades a 23-valent polysaccharide vaccine has been used, however, this vaccine does not give an adequate immune response in the risk groups i.e. small children and the elderly. Hence, conjugated vaccines have been created which elicit a T cell-dependent response leading to better protection in young children. However, only a limited number of capsular types have been included in the current vaccine PCV7, where seven out of the 91 serotypes are included and chosen because they are the most abundant serotypes that cause invasive pneumococcal disease in the USA. Therefore this vaccine does not protect against all pneumococci circulating in society and there is a risk for the emergence of non-vaccine types and serotype replacement. The vaccine was introduced in the USA in 2000 and has since been introduced in several countries in the Western world. Results from the USA show a significant decrease in invasive pneumococcal disease, a reduction of vaccine types in carriage and a herd effect in the adult population. However, recent data has shown an increase in non-vaccine types and serotype replacement in pneumococcal disease and in colonization. Furthermore, an increasing prevalence of pleural empyemas as a complication of CAP has been reported worldwide. A study from Israel showed that the prevalence of pleural empyemas increased in southern Israel before the introduction of PCV7 and that type 1 was the most common type [4]. This capsular serotype is not included in the current PCV7 and we need to follow the epidemiology since vaccination may impact on clinical presentation and epidemic trends. Industry is now interested in developing new affordable pneumococcal vaccines including either more serotypes in conjugated vaccines or

vaccines that have other targets such as the surface proteins which are present in most or all pneumococci. Candidate antigens include non-toxic derivatives of pneumolysin, surface proteins such as PsaA, and pilus proteins as well as choline binding proteins such as PspA and PspC, etc. (see below). At present there is no vaccine available for *Legionella* infections.

Both pneumococci and *Legionella* cause pneumonia, however their virulence strategies are as different as their lifestyles. Several virulence factors have been described for both bacteria. In the following we will describe some important features and factors involved in the disease process of each pathogen.

6.2
Streptococcus pneumoniae, a Devastating Pathogen, but also a Common Colonizer of the Upper Respiratory Tract

6.2.1
Epidemiology of Pneumococcal Disease and Carriage

Streptococcus pneumoniae is a major cause of milder respiratory tract infections such as otitis media and sinusitis, but also a major contributor to community-acquired pneumonia. Pneumococci are also a common cause of invasive diseases such as septicemia, a common complication of pneumonia, and meningitis. The introduction of penicillin (and other antimicrobial drugs) caused a dramatic reduction in the mortality of all pneumococcal diseases except that of meningitis. However, attack rates of pneumococcal diseases have not been reduced. Current estimates from the United States indicate that the rate of different pneumococcal diseases has not changed during the antibiotic era. The annual figures of about half a million cases of pneumonia, 50 000 cases of bacteremia, 3000–5000 cases of meningitis and a large fraction of the several million cases of otitis media caused by pneumococci, all remained essentially unchanged. The WHO estimates suggest that fatal pneumococcal infections contribute significantly (1–2 million cases) to the annual global mortality rate attributed to respiratory disease and is in the same range as the fatality rate of tuberculosis [3].

Despite being a devastating pathogen pneumococci are also common colonizers of the upper respiratory tract and up to 60–70% of children attending day-care centers may harbor these bacteria in the respiratory tract and be disease free. A major question is how these bacteria sometimes cause severe diseases while they are usually only harmless colonizers. To study the spread of pneumococci we used different classical typing methods and molecular techniques. As stated above pneumococci can be divided into at least 91 serotypes depending on their capsular polysaccharide structures, and an association between virulence and capsular type has been observed. Also, a non-encapsulated pneumococcus is regarded as non-virulent, but we have also found unencapsulated strains among clinical isolates from healthy carriers and from sterile sources in infected patients. To investigate differences in the potential of different pneumococci to cause invasive disease,

isolates have been collected from both invasive disease cases and carriers over the same time period and from the same geographic area in several studies around the world. In these studies certain serotypes have been found to be more invasive i.e. more prone to cause invasive disease (types 1, 4, 5, 7F, 14) when acquired, than others that are more weakly associated with invasive disease and mainly cause carriage (3, 6A, 19F etc) [5, 6]. Also, disease severity and disease type has been correlated with age, underlying disease, and capsular serotype for patients with invasive pneumococcal disease. These data suggest that strains of serotype with a high invasive disease potential (type 1 and 7F) not only have a high potential to cause invasive disease, but also are capable of causing disease in previously healthy individuals and evoking a high frequency of pneumonia [7]. These serotypes can therefore be regarded as primary pathogens that probably have a limited ecological reservoir, rare in carriage, and propagate in the population primarily by causing disease. Isolates belonging to these serotypes are represented by few clones, and antibiotic resistance is uncommon. In contrast, serotypes with a lower relative risk of causing invasive disease are more opportunistic, primarily affecting patients with underlying disease. Disease caused by the latter group, however, tends to be more severe, even in previously healthy individuals.

Certain serotypes have been associated with carriage in children but also with the development of antibiotic resistance, these include types 9V, 6B, 14, 19F, and 23F. The leading cause of acute otitis media (AOM) is pneumococci which account for 28–55% of AOM cases. The most abundant serotypes associated with AOM are the same as those found in carriage, i.e. types 6A, 6B, 9V, 14, 19F and 23F. In comparison to other pathogens causing AOM, pneumococci seem to evoke a more severe inflammatory response with higher serum interleukin levels.

However, it is not sufficiently accurate to use serotyping alone to study the spread and genetic relationships of these organisms, other molecular methods should also be employed such as restriction fragment analysis, e.g. PFGE (pulsed field gel electrophoresis) where the chromosomal DNA of the bacteria is cleaved with a restriction enzyme and thereafter separated on a pulsed field gel. Other methods include sequence-based techniques such as MLST (multi locus sequence typing) where parts of seven different house keeping genes are sequenced and compared. The data generated using these techniques suggest that it is not only the capsular type which is important for virulence but that other factors also play a role. Furthermore, using microarray technology based on whole pneumococcal genomes (we used the laboratory strains R6 [8] and TIGR4 [9], originally an invasive isolate from Norway) to study the presence/absence of genes in clinical isolates, we found that there is significant redundancy among virulence genes in cases where isolates that are perfectly capable of causing invasive disease may lack several virulence factors as identified by signature tagged mutagenesis (STM) and mice screens [10]. In this study 47 pneumococcal isolates comprising 13 serotypes, were compared and it was found that about 34% of the genes were variable including 95 genes previously shown by STM screens to be required for invasive disease in mice. Several of the genes that varied, known as Accessory genes, were localized to 41 Accessory Regions (ARs). The repertoire of ARs differed between clones and

even between isolates of the same clone. No unique pattern distinguished the most invasive clones from the others.

6.2.2 *In vivo* Studies of Pneumococcal Infections

Streptococcus pneumoniae is a human adapted pathogen, however several animal models have been established for virulence, vaccine efficacy, antibiotic resistance and therapeutic/drug efficacy studies both in immunocompetent and immunocompromised animals [11] Mice have become the most commonly used animal and mouse lines such as C57BL/6, BALB/c, DBA and CBA have been used for the analysis of pneumococcal pneumonia, meningitis and otitis media. Other animals that have been utilized include rats for studies of invasive disease and otitis media, rabbits for pneumococcal pneumonia, septicemia and meningitis studies, as well as chinchilla, gerbil, ferrets and guinea pigs for experimental otitis media studies.

In mouse models of pneumococcal pneumonia the mice are challenged with bacteria either intranasally or intratracheally. The intranasal aspiration model is most common as it closely mimics the natural route of infection in humans. Anesthetization of mice allows infection of the lower respiratory tract, while absence of anesthesia is used in colonization models. A lobar pneumonia is induced in the intratracheal inoculation model, while intranasal aspiration evokes a bronchopneumonia in mice. Parameters such as survival, bacterial counts in blood, lungs and other organs, levels of inflammation, histology and antibody responses can be measured and evaluated. Invasive septic models include inoculation of the bacteria either intraperitoneally (i.p.) or intravenously (i.v.). Experimental data has shown that some pneumococcal strains that were fatal after i.p. injection were avirulent by the i.v. route in mice. Hence, i.p. infections have been used to passage various strains in mice to render them more virulent. Rats have been especially valuable in sepsis studies in splenectomized and immunocompromised animals. Meningitis may appear in humans after pneumococcal colonization of the nasopharynx and subsequent pneumonia and septicemia, followed by traversal of the bacteria across the blood brain barrier. The bacteria may also pass via the olfactory nerve or nasopharynx/sinuses directly into the meninges [12]. Experimental meningitis models have mainly used rat and rabbit, but recent models have also included mice. In these models bacteria have been installed either directly via the intracerebral or intracisternal routes or inoculated intranasally or intraperitoneally. A drawback of the i.n. and i.p. routes is that a majority of the animals will succumb to a lethal infection due to sepsis without developing meningitis.

Several studies have been carried out to investigate the differences in virulence between pneumococcal strains [11, 13]. Virulence is largely dependent on the pneumococcal serotype. Typically strains of serotypes 2, 3, 4, 5 and 6 may cause a lethal disease in mice, while for example strains of types 14, 19 and 23 do not kill the mice after intranasal inoculation. However, it is not only the capsular serotype that is important but also other genetic factors and by undertaking

mutant analysis several researchers have found important virulence attributes among pneumococcal strains.

Susceptibility to pneumococcal infections also differs with the mouse strain used. We found that some clinical isolates evoked more sepsis in C57BL/6 mice than in BALB/c mice, but evoked more meningitis in BALB/c mice than in C57BL/6 mice [13]. Hence, host susceptibility is of utmost importance for disease outcome and will be discussed further below.

6.2.3 Pneumococci are Naturally Competent Bacteria

Streptococcus pneumoniae are naturally transformable organisms i.e. have the ability to take up free DNA from their surroundings and incorporate it into their genome by homologous recombination. Historically, pneumococci played an important role in demonstrating that DNA is the genetic material responsible for trait inheritance. In 1928 Griffith showed that avirulent pneumococci could become virulent by adding heat-killed virulent pneumococci. Later, in 1944 Avery and colleagues demonstrated that DNA is the factor that is transformed when non-encapsulated rough pneumococci become smooth, encapsulated and hence virulent.

The state of competence is a transient phenotype allowing competent bacteria to take up DNA from non-competent pneumococcal cells after lysis [14]. This phenotype also known as the X-state is induced by peptide pheromones through a quorum sensing mechanism. Thus far two main secreted competence-stimulating pheromones (CSPs) have been identified, CSP1 and CSP2 [15]. They interact with two membrane-associated histidine kinase receptors, Com D1 and ComD2, in the two-component regulatory system ComDE. ComDE detects extracellular CSP and initiates a signal leading to transcriptional activation of the competence regulon (*com* regulon), comprising 105–124 genes. However, only about 25% of these genes are required for transformation suggesting that the *com* regulon might also be involved in other functions. Binding of CSP to ComD leads to the induction of autophosphorylation of this receptor followed by the transfer of the phosphoryl group to ComE which activates transcription of *comX* and other early *com* (competence) genes such as *comAB*, *comCDE*, and *comW* [16]. The late *com* genes include those involved in the machinery for DNA uptake and processing. Induction of the competent state initiates release of DNA from a subfraction of the bacterial population. Why some bacteria do not respond to CSP is not known.

The biological role of competence is under investigation. There seems to be an association between competence and virulence and it has been shown that a mutant in *comD* is attenuated in a mouse model. Furthermore, it has been suggested that there is a link between transformation and the killing mechanisms of the bacteria known as fratricide, and that fratricide involves bacterial lysis [17, 18]

Another consequence of natural competence among pneumococci is that the bacteria have been shown to take up DNA from other organisms in their environment such as oral streptococci and co-colonizing pneumococci. Acquisition of

genes and gene fragments from other pneumococci and also from related commensal streptococci has resulted in mosaic genes encoding altered penicillin-binding proteins, which explains the emergence of resistance to penicillin among pneumococcal clinical isolates.

6.2.4 Pneumococcal Colonization of the Respiratory Tract

Pneumococci cause diseases sometimes with a lethal outcome but they are also commensals that colonize healthy individuals especially small children in whom the peak of colonization occurs at around 2–3 years of age. Person to person spread is thought to occur via the spread of droplets/aerosols. From its ecological niche, the nasopharynx, pneumococci are spread to the ears, lungs, blood and meninges causing disease. Hence, the prerequisite for disease is colonization. Many factors have been shown to be important for colonization [19]. These include phosporylcholine (ChoP) which is present in the cell wall components lipoteichoic acid and teichoic acid. ChoP mediates adherence to the receptor for the platelet-activating factor, PAF (platelet-activating factor receptor) and binds to CRP (C-reactive protein). Binding to the rPAF has been shown to mediate adherence to activated host cells and transcytosis of the blood brain barrier. Pneumococcal surface proteins that act as adhesins are choline-blinding proteins such as PspC (also called CbpA or SpsA) which is non-covalently bound to ChoP as well as LPXTG proteins including the pilus proteins (see below). Other factors that influence colonization include the capsule which inhibits the entrapment of the bacteria in the mucus and reduces opsonophagocytosis. Furthermore, enzymes such as the three exoglycosidases that are surface exposed, NanA (a neuraminidase), BgaA (a beta-glucosidase) and SrtH (a beta-N- glucosaminidase) affect colonization by cleaving terminal sugars from human glycoconjugates, thereby possibly exposing receptors for adherence. Hyaloronidase may facilitate the spread of the bacteria into the tissue and components like PavA (pneumococcal adhesion and virulence A) and enolase might bind the bacteria to extracellular matrix proteins such as fibronectin and plasminogen.

The human respiratory tract is the habitat for more than 500 different microbial species. Hence, pneumococci encounter many other organisms in their environment and co-colonization by other species has been shown to affect pneumococcal colonization. Common colonizers which may also be pathogens, in the upper respiratory tract include *Haemophilus influenzae* and *Staphylococcus aureus*. An experimental model of co-colonization with *Haemophilus influenzae* resulted in the clearance of pneumococci from the upper respiratory tract in mice, probably due to enhanced opsonophagocytic killing mediated by the increased recruitment of neutrophils [20]. A suggested mechanism for this interaction is that the pore-forming toxin pneumolysin in pneumococci helps the peptidoglycan of *H. influenzae* to be recognized in the host cytoplasm by the host cytoplasmic peptidoglycan receptor Nod1 [21]. Also, *Staphylococcus aureus* co-colonization has been shown to have an impact on pneumococcal colonization. Clinical studies in humans have

revealed an inverse relationship between nasopharyngeal carriage of *S. aureus* and vaccine-type strains of *S. pneumoniae* [22, 23], suggesting natural competition between these bacterial species in the nasopharyngeal niche [24]. Furthermore, despite vaccination with the seven-valent conjugated pneumococcal vaccine a trend showing an increase in *S. aureus*-mediated acute otits media and bacteremia in young children was observed [25, 26].

However, the details of the mechanism which determine the outcome of co-colonization with different species remain unclear. Recent data suggest that competition also occurs between different pneumococcal strains and that bacteriocins, pneumocinMN encoded by the *blpMN* genes, might play an important role [27]. Pneumococci can be distinguished into two different morphologies: transparent and opaque phenotypes. The transparent phenotype seems to be expressed mainly during colonization and the opaque mainly after colonization. Transparent colonies have a less robust capsule and an increased concentration of teichoic acid (see below, cell wall components) together with an increased propensity for autolysis (LytA see below) as compared to opaque variants. The same research group has now noted that transparent variants provide a competitive advantage over opaque variants since they do not produce pneumocinMN. Furthermore, they found that the differential regulation in the opacity variants is driven by the two-component regulatory system CiaRH via its regulation of the serine protease, HtrA [28].

Many bacterial infections have been shown to involve biofilm production which is also a characteristic of pneumococci. Using a transposon library Munoz *et al.* found 23 biofilm-deficient mutants in the TIGR4 background that were also impaired in their colonization of the nasopharynx in mice. The authors therefore drew the conclusion that there is a correlation between defective biofilm production and colonization *in vivo* [29]. Interestingly one suggested adhesin, the LPxTG protein RrgA, which is a subcomponent of a pneumococcal pilus (see below), was shown to be involved in biofilm formation. In a recent study of virulence *in vivo*, Oggioni *et al.* found that during sepsis the growth of pneumococci resembled that of plankton, whilst in pneumonia and meningitis pneumococci are in a biofilm-like state [30].

6.2.5
Synergism between Influenza Virus and Pneumococci

It is well known that viral respiratory tract infections lead to a predisposition for secondary bacterial infections. So far epidemiological support for this interaction has only been found between influenza virus and pneumococci [31]. Current evidence indicates that co-infections with pneumococci during the influenza pandemic (1918–1919) was largely responsible for the ~50 million deaths that occurred. As a result, vaccination against pneumococcal infections has now been suggested as a strategy to avoid future deaths during influenza pandemics. A study conducted in South Africa supports these findings as vaccination against pneumococcal infection with a nine-valent conjugated vaccine reduced the number of viral pneumonia cases caused by RSV, influenza and parainfluenza viruses [32].

The exact details of the mechanisms behind this interaction are still unknown and extensive research is ongoing. The classical dogma has been that the virus damages the epithelial barrier thereby exposing extracellular matrix components to which the bacteria can adhere. In support of this, a mouse-adapted virus in an experimental murine model was shown to cause severe epithelial damage to the lungs and death, and a secondary infection with pneumococci led to an increased bacterial load in the lungs as a result of the bacteria attaching to regions that had already been damaged by the virus [33]. However, a non-mouse-adapted virus with lower pathogenic potential did not cause pathological damage to the respiratory epithelium and yet the bacteria persisted in the lungs for some time. The virulence of the virus alone is usually not sufficient to cause death but exceptions include the 1918 pandemic influenza virus strain and the H5N1 avian influenza strain. Hence, the co-infection mortality may also be dependent on the strain of the virus. The virus strain may also mediate changes in the airway functions and may up-regulate and enhance exposure to receptors that affect bacterial binding. Both pneumococci and influenza viruses possess neuraminidases (also known as sialidases). The viral neuraminidase cleaves the terminal sialic acid residues leading to release of viruses from infected cells. Pneumococcal neuraminidases include NanA, NanB and NanC, and NanA and NanB have been shown to be involved in pathogenesis (only NanA contains an LPxTG motif (see below) and might be involved in adherence), and both promote bacterial survival in the respiratory tract and bloodstream. Inhibitors of influenza virus neuraminidase have been shown to prevent secondary bacterial infections in experimental models. Vaccination against influenza has also been shown to decrease the prevalence of secondary bacterial pneumonia.

Recently, an interesting paper published in *Nature Medicine* showed that pulmonary interferon-gamma (IFN-gamma) produced during T cell responses to influenza virus infection in mice, inhibits bacterial clearance from the lungs by alveolar macrophages and that this suppression of phagocytosis leads to increased susceptibility to secondary pneumococcal infection [34].

6.2.6
Some Important Bacterial Virulence Factors and their Interactions with the Host

Several factors important in pneumococcal pathogenesis have been described with regard to both the bacteria and the host. A few of these factors will be described here including some that are promising as vaccine candidates, focusing on adherence molecules, the cytolytic toxin pneumolysin, as well as the innate immune system recognizing molecular patterns of the organisms.

The **polysaccharide capsule** is a major virulence characteristic and because of differences in their structure at least 91 different variants can be distinguished hence dividing the bacteria into serotypes (see above). Most of the capsules have recently been sequenced and 88 serotypes have been shown to be synthesized by the Wzy-dependant pathway where the *cps* loci encoding the serotypes are localized at the same chromosomal locus between the genes *dexB* and *aliA* [35]. Capsule production is the major determinant of pneumococcal virulence as the capsule is

anti-phagoytic and affects complement deposition on the bacterial surface. The capsule is about 200–400 nm thick and with the exception of the type 3 capsule and also possibly other capsules, it is covalently bound to the outer surface of the cell wall peptidoglycan. For unknown reasons capsule production is reduced upon contact with epithelial cells [36].

The first step in pneumococcal pathogenesis is colonization followed by the spread to other organs within the body and transmission through mucosal barriers leading to dissemination, as described above. Hence, it is important for the bacteria to have the ability to adhere to host cells such as epithelial cells in the respiratory tract. Adherence molecules include surface-exposed proteins and MSCRAMMs (microbial surface components recognizing adhesive matrix molecules) which mediate binding to host cells and extracellular matrix proteins via different receptors. A number of studies have focused on the surface-located so-called choline binding proteins (CBPs). Up to 16 CBPs have been described so far, including PspA, PspC (CbpA or SpsA), as well as the four cell wall hydrolases LytA (*N*-acetylmuramoyl-*L*-alanine amidase), LytB (beta-*N*-acetylglucosamidase), LytC (lysozyme) and a phosphorylcholine esterase (Pce or CbpE) [37]. All CBPs share homologies in their conserved choline-binding domain that is located in the C-terminal end of the protein. The proteins are bound non-covalently by this domain to the phosphorylcholine of lipoteichoic acids and teichoic acids in the cell wall.

PspA (pneumococcal surface protein A) has been shown to be involved in immune evasion and inhibits activation and deposition of complement C3 through the classical pathway as well as via the alternative pathway by reducing C3b deposition. It also binds to the iron transporter lactoferrin, thereby preventing the bactericidal effect of this human protein [38]. Loss of PspA increases complement-receptor-mediated clearance of pneumococci [39]. The *pspA* gene is highly variable and PspA can be divided into three major families that can be further divided into six different clades depending on the sequences in the N termini of clinical isolates [40]. Loss of PspA reduces virulence in a murine model of bacteremia in some strains, however the influence on virulence has been somewhat debated. PspA has been suggested as a candidate in a protein-based vaccine since it is protective in animals and induces protective antibodies in humans.

PspC binds to pIgR (polymeric immunoblobulin receptor for adherence) and promotes adherence and uptake into nasopharyngeal cells. A mutant in *pspC* has been shown to reduce adherence to cells and to reduce colonization in an infant rat model [41]. Microarray analysis indicates that *pspC* is up-regulated during attachment to nasopharyngeal epithelial cells, but not in the blood or cerebrospinal fluid. PspC binds to complement protein C3 and factor H and is thereby important for evasion of complement. The PspC-like protein Hic binds to factor H and contains an LPxTG motif. Hic is mainly found in strains of serotype 3, and the protein anchors to the cell wall in a sortase-dependent manner. Recruitment of Hic has been shown to prevent activation of C3b and complement-mediated opsonophagocytosis. Both PspC and Hic have been shown to promote increased survival of pneumococci in a systemic mouse model [42]. However, it has recently been shown that virulence in a systemic and pulmonary mouse model depends on the

pneumococcal strain used [43]. PspC has also been recognized as a potential vaccine candidate.

The peptidoglycan hydrolases are enzymes that cleave the covalent bonds of the cell wall and in some cases eventually cause lysis and death of the cell, in this case these enzymes are then known as autolysins. The major autolysin of pneumococci is **LytA**, an amidase that cleaves the *N*-acetylmuramoyl-*L*-alanine bond of the peptidoglycan. LytA has been implicated in pathogenesis as an important factor since its activity leads to the release of cell wall fragments such as teichoic acid, lipoteichoic acid and peptidoglycan as well as pneumolysin after bacterial death which may induce pro-inflammatory responses (see below). LytA has been shown to be important for virulence and to contribute to bacterial replication in the lungs and translocation to the bloodstream [44]. LytA has been suggested as a vaccine candidate. **LytB** is a glucosaminidase and is the key enzyme in the separation of daughter cells. It is believed to play a critical pathogenic role, facilitating bacterial spread during infection and has been suggested as a putative vaccine target [45].

LytC is a lyzosyme which carries out a slow autolysis with an optimum activity at 30°C. **Pce** or **CbpE** is an esterase whose structure has been determined [42]. LytB, LytC and Pce have been shown to affect colonization in an infant rat model, however these enzymes did not influence virulence in a sepsis model [46],

CbpD, a competence-stimulating-peptide-inducible protein, is a serine protease, and has been shown to play a role in colonization [46].

Several lipoproteins have been suggested to be involved in pneumococcal virulence such as the peptide isomerases PpmA and SlrA, as well as PsaA (pneumococcal surface antigen A), PiaA (pneumococcal iron acquisition A), and PiuA (pneumococcal iron uptake A). **PsaA** is detected on all known serotypes and is a metal-binding lipoprotein with specificity for Mn^{2+} and Zn^{2+}. It is the substrate-binding lipoprotein of an ABC-type manganese-transport system [47]. ABC transporters have been shown to play a major role in bacterial adherence and virulence. PsaA mediates adherence to E-cadherin (cell–cell junction protein in respiratory epithelium) and mutations in *psaA* reduce adherence to host cells. Furthermore, deletion of *psaA* leads to reduced virulence in murine models of pneumonia, bacteremia and colonization and attenuation in intraperitoneal mouse infection models. In microarray studies *psaA* has been found to be up-regulated during attachment to nasopharyngeal cells [48]. However, the adhesive effect might partly be due to effects on the expression of other pneumococcal genes such as adhesins. PsaA is immunogenic and antibodies to this protein have been found after nasopharyngeal colonization suggesting PsaA to be a vaccine candidate [49].

The pneumococcal genome contains about 13 proteins having **LPxTG motifs**. These proteins are covalently anchored to the cell wall after cleavage of the LPxTG sequence by a transpeptidase known as a sortase. Recently, we found that three of these proteins, **RrgA**, **RrgB** and **RrgC** encode a pilus-like structure on the surface of pneumococci [50]. The genes encoding these proteins, *rrgA, rrgB* and *rrgC* are all located in a pathogenicity island (Accessory Region) called the *rlrA* islet. The islet was thus named because of the positive regulator *rlrA* located within the operon. Inside the operon there are also three genes encoding three sortases, SrtB,

SrtC and SrtD, as well as two IS elements located at either end of the operon respectively. Outside the operon there is a negative regulator called *mgrA*. We showed that the pneumococcal pilus is important for adhesion to lung epithelial cells *in vitro* and for colonization, and virulence in a mouse model of pneumonia and septicemia. Furthermore, the pilus was found to induce an inflammatory response (TNF) as determined in an intraperitoneal model of infection [50]. We and others have determined the composition of the pilus [51, 52]. The major stalk of the pilus is composed of RrgB decorated with the minor subunits, RrgA and RrgC. RrgC is mainly found at the tip of the pilus or along the stalk and RrgA also along the stalk, but also along the entire cell surface [51]. Recent data have shown that the three pilus-associated sortases, SrtB, SrtC and SrtD, are required for biogenesis of the pilus [51]. Expression of SrtB alone was sufficient to covalently associate the RrgB subunits with one another and to link RrgA and RrgC into the pilus polymer. SrtC was redundant to SrtB in allowing RrgB polymerization and in linking RrgA to the RrgB filament. However, SrtC could not incorporate RrgC. SrtD on the other hand could not mediate RrgB polymerization alone. In conclusion, the data suggest that both SrtB and SrtC act as pilus subunit polymerases, with SrtB processing all three pilus subunit proteins while SrtC can only process RrgB and RrgA. In contrast, SrtD does not act as a pilus subunit polymerase. The respective crystal structures for all three sortases have now been determined. Furthermore, we investigated which subcomponent was mainly responsible for adherence and virulence [53, 54]. The minor pilin subunit RrgA was discovered to be the major protein responsible for adhesion to lung epithelial cells and also important for colonization *in vivo* [55].

Epidemiological analysis of clinical pneumococcal isolates revealed that the pilus is present in specific clonal types and it has been suggested to be one of the reasons for the successful spread of certain antibiotic-resistant clones across the world [56] Since the pilus is present mainly among clinical isolates from carriage and those with antibiotic-resistant determinants and it provides protection in animal models, it has been suggested as a vaccine candidate in a multi-component pneumococcal vaccine. Recently, another pilus was found using publicly available sequenced pneumococcal genomes. This pilus encoded by a pathogenicity islet known as PI-2 consists of *pitA, sipA,* and *pitB,* as well as two sortases, *srtG1*, and *srtG2,* and is present in some serotypes which lack the first pilus described above, such as types 1, 2, and 7F, but is also present in type 19F isolates. *In vitro* experiments provide evidence that this pilus is also involved in adherence. Thus, pneumococci encode at least two types of pili that play a role in the initial host cell contact with the respiratory tract and are potential antigens for inclusion in a new generation of pneumococcal vaccines [57].

Among the other proteins carrying LPxTG motifs which anchor the proteins to the cell wall, are four zinc metalloproteases including **IgA1-protease**, **ZmpB**, **ZmpC** and **ZmpD**. IgA protease, ZmpB and ZmpC have been classified as STM (signature tagged mutagenesis) genes, i.e. important for virulence in lung infections and bacteremia. Weiser *et al*. has also shown that cleavage of IgA leads to enhanced adherence to host cells [58]. ZmpC (Immunoglobulin A1 protease) is a

bacterial protease and it cleaves MMP-9 (human matrix metalloproteinase 9). Oggioni *et al*. has reported that inactivation of the *zmpC* gene in a 19F strain led to impaired virulence in a pneumonia model [59].Using microarray whole genome analysis we found that many clinical isolates lack the *zmpC* gene (unpublished data).

A novel serine-rich repeat protein known as **PsrP (SP1772)** has recently been shown to be a pneumococcal adhesin for A549 cells. It is encoded by the pathogenicity islet *psrP-secY2A2*. PsrP was shown to be required for persistence of bacteria in the lungs, but not for colonization of the nasopharynx. Furthermore, it was found to be a protective antigen [60].

Pneumolysin is a cholesterol-dependent cytolysin that has the capacity to form pores in eukaryotic cell membranes due to its hemolytic activity, but it is also able to activate complement via the classical pathway. Pneumolysin is a 53-kDa protein composed of 471 amino acids and contains only one cysteine residue that is required for optimal hemolytic activity and the overall structure. The protein is negatively charged and it resides in the cytoplasm rather than being exposed on the surface of the bacteria. It is released upon lysis of the bacteria which has traditionally been believed to occur during the stationary phase as a result of the action of autolysins. However, pneumolysin can be released in the log-phase of some strains. Pneumolysin lacks an *N*-terminal secretion signal sequence implying that it cannot be secreted via the sec-dependent pathway, suggesting a putative alternative secretion system for this protein.

Pneumolysin is found in most pneumococcal isolates and shows some genetic variation among clinical isolates. It has been reported that type 1 isolates of ST306 lack a functional pneumolysin due to mutations [61]. Also, type 7F and type 8 strains have been shown to encode a pneumolysin with reduced hemolytic activity. Serotype 1 and 8 pneumococci are known to be associated with outbreaks of invasive disease [62]. The clinical implications of this reduced activity remain to be elucidated.

Pneumolysin is a potent virulence factor and inhibits the beating action of the cilia on respiratory epithelium and is suggested to be crucial for pneumococcal virulence in pneumonia. However, it is likely that this virulence might be strain specific. The protein is cytolytic, but also activates several genes in eukaryotic cells such as those encoding chemokines and cytokines thus leading to an inflammatory response and the TLR4 receptor (see below) has been shown to recognize this protein. In mice, pneumolysin influences bacterial persistence in the lungs and facilitates replication in and spread to the blood. Hence, intranasal inoculation of pneumolysin-deficient mutants into mice led to increased bacterial clearance from the lungs and increased survival as compared to the wild-type. The hemolytic activity contributes to the recruitment of neutrophils and the complement activation to T-cell recruitment, all important factors in the host response against pneumococci [63]. Programmed cell death or apoptosis is a feature known to occur in pneumococcal pneumonia and meningitis [64]. In combination with hydrogen peroxide (see below) pneumolysin induces apoptosis. In addition to direct cell cytotoxicity of the neurones, endothelial cells and microglia together with the

recruitment of inflammatory cells, apoptosis of cells in the central nervous system contributes to the morbidity and sequelae such as deafness, observed in pneumococcal meningitis. However, apoptosis during pneumococcal infection of the respiratory tract has been associated with improved outcome [65, 66]. Pneumolysin confers protection in mice and hence has been suggested as a vaccine candidate in a protein-based vaccine.

Lyt A has been described briefly above as an amidase leading to bacterial death. **Hydrogen peroxide** produced by the enzyme pyruvate oxidase (SpxB) also kills bacteria in a manner resembling apoptosis in eukaryotic cells according to Regev-Yochly and colleagues [67]. In this study, the authors claim that spontaneous pneumococcal death in the stationary phase is due to hydrogen peroxide, and not to LytA. Furthermore, they show that the presence of SpxB confers an advantage in a colonization infant rat model. It has also been shown that SpxB contributes to bacterial replication in the lungs and translocation to the bloodstream [44].

6.2.7 Host Defenses against Invading Microbes

Defenses against pneumococci in the host include both the innate and adaptive immunity with recruitment of phagocytic cells such as neutrophils, macrophages and dendritic cells to the site of infection, as well as induction of the complement cascade, activation of receptors such as the Toll-like receptors (TLRs) and the NOD (nucleotide-binding oligomerization domains) receptors which recognize invading microbes, together leading to an inflammatory reaction, T-cell activation and antibody response. We will focus here on the early innate immune response particularly the role of TLRs in pneumococcal infections and the production of so-called Neutrophil extracellular traps (NETs) [68] and their involvement in pneumococcal disease.

TLRs recognize different bacterial components leading to a signaling cascade that leads to the transcription of genes via adaptor proteins such as the MyD88 protein, thus inducing production of pro-inflammatory cytokines and chemokines, resulting in inflammation. Components that may be recognized by TLRs include for example the cell wall. Pneumococci have a cell wall surrounding the cytoplasmic membrane which consists of a thick layer of peptidoglycan (also known as murein) and a polymer of carbohydrates (*N*-acetylmuramic acid and *N*-acetylglucosamine) which are cross-linked by peptide bonds [69].The cell wall also consists of teichoic acids and lipoteoichoic acids, the latter being anchored to the cytoplasmic membrane via lipid moieties and the former being covalently linked to the peptidoglycan. Choline residues of teichoic acids provide binding sites for choline-binding proteins. Several TLRs have been suggested to be involved in pneumococcal infections. The adaptor protein myeloid differentiation factor 88, **MyD88**, has been found to be a key player in pneumococcal infections. Both local and systemic inflammatory responses to pneumococci depends on the presence of MyD88 to clear bacterial colonization of the upper respiratory tract and to prevent pulmonary and systemic infection in mice and a strong correlation has been described

between enhanced bacterial growth in the bloodstream of MyD88-deficient mice and the inability to lower the serum iron concentration in response to infection [70]. **TLR2** may present as a heterodimer in association with TLR1 or TLR6 and recognizes lipoproteins and lipoteichoic acid in the cell wall and possibly even peptidoglycans (data are controversial). TLR2 has been shown to play a role in a meningitis model however its involvement in pneumonia has so far only been found to be marginal. **TLR1** and **TLR6** seem to be redundant or play only a minor role in pneumococcal infections as evidenced by the available data. Pneumolysin has been found to interact with **TLR4** [71], a TLR that recognize the LPS of Gram-negative bacteria. Finally, in a study by us using an intranasal model of infection we found that of the TLRs tested (TLR1, 2, 4, 6, and TLR9) only TLR9 played a non-redundant role. **TLR9** recognizes unmethylated CpG DNA and was shown to play a protective role in the lungs at an early stage of infection prior to the involvement of circulating inflammatory cells. Alveolar and bone marrow-derived macrophages deficient in TLR9, showed an impaired ability to phagocytose and kill pneumococcal cells [72].

Other receptors shown to be involved in pneumococcal pathogenesis include SIGNR1, a C-type lectin expressed on macrophages. SIGNR1 has been shown to mediate the uptake of both purified capsular polysaccharides and pneumococci in the spleen. Also, the scavenger receptor Marco has been reported to affect pneumococcal disease. In a murine model of pneumococcal pneumonia, $MARCO^{(-/-)}$ mice displayed an impaired ability to clear bacteria from the lungs, increased pulmonary inflammation and cytokine release, and diminished survival [73].

Polymorphonuclear leukocytes (neutrophils) are important players in the first line of defense against invading microbes. Neutrophils phagocytose bacteria and kill them intracellularly. A new mechanism known as neutrophil extracellular traps (NETs) was reported in 2004 to explain the interaction of bacterial cells with neutrophils [68]. NETs are formed when neutrophils are exposed to bacteria and they are composed of a backbone of DNA, histones, and enzymes such as neutrophil elastase and myeloperoxidase. Zychlinsky *et al*. showed that neutrophils may trap, kill and disarm salmonella, shigella, and staphylococci by forming these NETs [68]. We have shown that pneumococci may also be trapped in NETs but unlike the other bacteria mentioned they are not killed [74]. However, we found that expression of the capsule significantly reduced the number of bacteria trapped by NETs [75]. Pneumococci contain the *dlt* operon that mediates the incorporation of D-alanine residues into lipoteochoic acids, thereby introducing positive charge. A mutation in *dltA* in a non-encapsulated pneumococcus rendered the organism sensitive to killing by antimicrobial components present in NETs, but the encapsulated *dltA* mutant remained resistant to NET-mediated killing. Furthermore, the encapsulated *dltA*-mutant strain was out-competed by the wild-type upon invasion of the lungs and bloodstream in a murine model of pneumococcal pneumonia. This suggests a non-redundant role for D-alanylation of lipoteichoic acid in pneumococcal virulence at the early stage of invasive disease when capsule expression has been shown to be low. Moreover, we found that encapsulated pneumococci are sensitive to killing by a human neutrophil granule extract composed of the

alpha-defensins HNP1-3 [76]. Surprisingly, non-encapsulated, non-virulent pneumococci were significantly less sensitive to alpha-defensins. Hence, alpha-defensins in the phagolysosome of neutrophils possibly contribute to intracellular killing after antibody-mediated opsonophagocytosis of encapsulated pneumococci [75].

6.2.8
Pneumococcal Genomes

Several pneumococcal genomes have been sequenced. The laboratory strain TIGR4 serotype 4, originally an invasive clinical isolate from Norway, was the first to be sequenced [9], followed by the laboratory strains R6 [8] that has been used in several *in vitro* studies on pneumococcal pathogenesis and function. The genomic DNA consists of a single circular chromosome with a relatively low G+C content of about 40% [9]. Recently many more genomes have been sequenced and in the study by Hiller *et al*. nine publicly available pneumococcal genomes were compared with eight sequenced nasopharyngeal strains (of types/groups: 1, 2, 3, 4, 6, 9, 11,14, 19, 23) [77]. These authors came to the conclusion that about 46% of the gene clusters was conserved among all these 17 strains and that the majority of the gene clusters, 1716 or 54%, were not found in all strains. Using a modeling technique they proposed that the *S. pneumoniae* supragenome consists of more than 5000 orthologous clusters and that 99% of these clusters can be identified if 33 representative genomes are sequenced. Today when whole genome sequencing is becoming cheaper and more accessible we will witness an explosion of sequencing of clinical isolates from different diseases with the aim of correlating genome content, virulence factors and pathogenesis. Comparisons of sequence data from multiple isolates of the same species is also in use by vaccine companies in their search for novel vaccine candidates and is known as pan-genomic reverse vaccinology [78].

6.3
Legionella pneumophila, an Environmental Bacterium but also a Cause of Severe Sporadic and Epidemic Pneumonia

6.3.1
Epidemiology of Legionnaires' Disease and Environmental Reservoir

The diseases and symptoms caused by *Legionella* are varied. A mild form of *Legionella* infection is Pontiac fever which is a self-limiting flu-like illness. Symptoms of Pontiac fever include pyrexia, headache, myalgia and malaise. In contrast, Legionnaires' disease or legionellosis is a severe pneumonia that results in multisystem disease [79, 80]. Legionnaires' disease (LD) occurs as sporadic cases or as outbreaks and is either community or nosocomially acquired. The clinical features of LD vary and range from malaise, cough, chills, dyspnea, headache and chest

pain, to diarrhea. One remarkable feature of LD is the range of extrapulmonary manifestations including a variety of central nervous systems manifestations, lethargy, confusion, hallucinations, hyperactive reflexes or urinary retention [81]. Risk factors for LD are underlying diseases such as diabetes, cancer, AIDS or end-renal stage disease. Further risk factors are older age, male sex, immunosuppression and smoking [82]. Community-acquired infection accounts for the largest proportion of cases [83] and *Legionella* accounts for 4–20% of community acquired pneumoniae [84]. According to the CDC the case fatality rate for community acquired and nosocomial LD ranges from 5 to 40% (http://www.cdc.gov/legionella/top10.htm). However, mortality from the disease has been decreasing over recent years, probably due to earlier detection and improved treatment [85].

Water is the major reservoir for these bacteria worldwide and in addition to natural water environments high concentrations of *Legionella* are often detected in man-made hot-water systems [86]. The survival and spread of the bacterium depends on its ability to replicate inside eukaryotic phagocytic cells such as *Acanthamoeba castellanii* and other protozoans. Despite the presence of many different species of *Legionella* in water reservoirs, over 50 species and 70 serogroups have been identified to date [86]. The vast majority of human disease is caused by a single serogroup (Sg) of a single species, namely *L. pneumophila* Sg1, which is responsible for about 84% of all cases worldwide [87]. Little is known about the distribution of *Legionella* species and serogroups in the environment. A study investigating 259 clinical and 3128 environmental strains isolated in France from 2001–2002 showed that *L. pneumophila* Sg1 accounts for 28.2% of environmental *Legionella* in contrast to 95.4% among clinical isolates [88]. In a recent survey to investigate the distribution of *L. pneumophila* in England and Wales carried out between January 2000 and March 2008, 167 clinical and 276 environmental isolates of *L. pneumophila* obtained by routine sampling of 'managed' water systems were characterized; the results demonstrated that of the clinical isolates, 97.6% were *L. pneumophila* Sg1, compared with only 55.8% of the environmental isolates [89]. Thus the distribution of environmental *L. pneumophila* Sg1 differs significantly from that of clinical *L. pneumophila* Sg1 strains. Furthermore, species like *Legionella anisa*, *Legionella dumoffii* or *Legionella feeleii* relatively frequently colonize water distribution systems but are rarely implicated in human disease [88–90]. From these reports it can be hypothesized that the high percentage of *L. pneumophila* Sg1 strains found in human disease is not due to their predominance in the environment, but is rather connected with the greater virulence of these strains with regard to man. A slightly different distribution is present in Australia and New Zealand, where *L. pneumophila* Sg1 accounts for 45.7% of community-acquired legionellosis, and *Legionella longbeachae*, which is frequently isolated from potting soil [91] accounts for 30.4% of cases [87].

Although there is a variety of typing methods available for the analysis of the *L. pneumophila* species, clinical and environmental isolates cannot be distinguished by these methods [92, 93]. Similarly, the genetic factors responsible for the greater ability of *L. pneumophila* Sg1 to cause LD are not yet known. Recently, comparative genomic hybridization (CGH) using DNA arrays, was applied in the study over

200 clinical and environmental *L. pneumophila* strains with the aim of identifying any correlations between functional traits, phylogeny and epidemiological characteristics [94]. No correlations between genetic characteristics and the source of isolation were found. However one specific clone, *L. pneumophila* strain Paris [95], was identified as being distributed worldwide and causing sporadic cases and epidemics [94]. A correlation between specific clones and their ecological niche has also been observed, as strains isolated from different cooling towers in Japan revealed the same PFGE profile and sequence type [96]. Later analysis showed that this 'Japanese cooling tower clone' shared its PFGE profile and sequence type with *L. pneumophila* strain Paris. Recent studies on different groups of *Legionella* have shown that only a few clones seem to be responsible for most epidemic and sporadic disease [89, 94, 96–98]. Thus, it seems that generally only certain clones of *L. pneumophila* cause disease in humans.

Legionellosis has emerged in the second half of the 20th century partly due to human alterations of the environment. Indeed, thermally altered aquatic environments can shift the balance between protozoa and bacteria resulting in rapid multiplication of *Legionellae* to infectious concentrations. Whether adaptation to a specific host or the ability to multiply in different protozoan hosts is correlated with the virulence and epidemiologic prevalence of *L. pneumophila* poses an interesting question.

6.3.2
The Dot/Icm Type-IV Secretion System Central to Pathogenesis of *Legionella*

The importance of protein secretion for *L. pneumophila* is highlighted by the presence of a wide variety of secretion systems. The two major secretion systems known to be involved in virulence are the Dot/Icm type-IV and the Lsp type II systems. However, *L. pneumophila* possesses several additional secretion systems including a second type-IV system termed Lvh, a type I secretion system known as Lss, a twin arginine translocation (TAT) pathway and several *tra*-like systems. In addition to these conserved secretory pathways, some *L. pneumophila* strains also encode a type-V secretion system.

The Dot/Icm type IV secretion systems of *L. pneumophila* is required for intracellular growth in human macrophages as well as in amoebae as it delivers a large cohort of bacterial effectors to the host cytosol that modulate host processes to the pathogen's advantage. This macromolecular complex is encoded by 26 genes whose individual functions are only partly understood [99]. To date, over 80 effectors have been identified by a variety of genetic, biochemical, and cell biological approaches, yet the function of the majority of these substrates remains undefined [100, 101]. The first effector of the Dot/Icm system to be characterized was RalF which is required for localization of the host protein ARF-1, a key regulator of vesicle trafficking from the endoplasmic reticulum to the phagosomes [102]. LidA, another substrate involved in recruitment of vesicles during vacuole biogenesis and in maintaining the integrity of the Dot/Icm complex [103] and LepA and LepB which are involved in egress of *Legionella* from the vacuole during amoeba

infection [104] were discovered later. Candidate effector proteins known as SidA–G were identified in 2004 using a two-hybrid screen with IcmG/DotF as bait followed by a screen of proteins transferred inter-bacterially with a Cre/loxP-based protein translocation assay [105].

Further functional characterization of these effector proteins has shown that they are indeed important in modulating host cell functions during intracellular replication of *L. pneumophila*. Two independent studies showed that SidM/DrrA is a guanosine nucleotide exchange factor. Enhanced by LidA, SidM/DrrA recruits Rab1 (a small host GTPase regulating ER-to-Golgi traffic) to *Legionella*-containing vacuoles [106, 107]. GDP-bound Rabs are maintained in an inactive state by a GDP association inhibitor (GDI) that prevents their spontaneous activation. Rabs are released from GDI by a guanine nucleotide dissociation inhibitor displacement factor (GDF) before their recruitment to the membrane and activation by GEFs. DrrA/SidM is characterized by two distinct regions: the N-terminal moiety recruits Rab1 to LCVs membranes and functions as a GDF while the C-terminal end, characterized by highly specific Rab1-GEF activity, activates Rab1 [108]. Weber and colleagues [109] proposed that *L. pneumophila* modulates host cell phosphoinositide metabolism and exploits the Golgi lipid second messenger phosphatidylinositol(4) phosphate (PI(4)P) to anchor secreted proteins to the *Legionella*-containing vacuole (LCV). SidC and SdcA have also been implicated in this process: SidC anchors to the membrane of the LCVs by specifically binding to phosphatidylinositol-4 phosphate (PtdIns(4)P) [109]. It was recently shown that SidM/DrrA is the predominant PtdIns(4)P-binding protein of *L. pneumophila* [110]. Thus *L. pneumophila* exploits PtdIns(4)P produced by PI4K IIIss to anchor the effectors SidC and SidM to LCVs. Furthermore SidJ is required for the efficient recruitment of endoplasmic reticulum proteins to the bacterial phagosome and SidF is implicated in the inhibition of macrophage apoptosis by targeting pro-death members of the Bcl2 protein family [111].

A complicating issue in the understanding of the precise functions of *L. pneumophila* Dot/Icm substrates arises from the fact that individual deletions of most substrate genes have little or no impact on the intracellular growth of the bacterium, probably due to considerable functional redundancy among different substrates towards host cell targets. In addition, genetic studies indicate that multiple vesicle trafficking pathways are directed towards the LCV, suggesting that redundancy extends to the host as well as the bacterium [112]. Therefore, alternative systems for generating effector-dependent phenotypes have been used. Ectopic expression in the budding yeast *Saccharomyces cerevisiae* has recently emerged as a powerful tool for both the identification and characterization of secreted bacterial effector proteins [113]. Importantly, the generation of yeast phenotypes by bacterial effector proteins is specific as non-translocated bacterial proteins rarely produce yeast phenotypes [114]. Yeast expression has been utilized to identify and characterize *L. pneumophila* Dot/Icm substrates using libraries constructed from randomly digested *L. pneumophila* genomic DNA. This approach identified VipA, VipD and VipF, which are vacuole protein sorting inhibitor proteins (Vip) that inhibit lysosomal protein trafficking by different mechanisms [115] and WipA,

WipB, YlfA (yeast lethal factor A), YlfB and six additional proteins containing leucine-rich repeats and/or coiled-coil domains also translocated via the Dot/Icm secretion system [116–118]. Recently a large-scale screen of a defined set of 127 confirmed or candidate Dot/Icm substrates for their effect on host cell processes using yeast as a model system was undertaken [119]. Expression of 79 candidates caused significant yeast growth defects, indicating that these proteins impact essential host cell pathways. Notably, a group of 21 candidates interfered with the trafficking of secretory proteins to the yeast vacuole. Three candidates that caused yeast secretory defects (SetA, Ceg19 and Ceg9) were investigated further. These proteins impinged upon vesicle trafficking at distinct stages and produced signals that allowed translocation into host cells by the Dot/Icm system. Ectopically produced SetA, Ceg19 and Ceg9 localized to secretory organelles in mammalian cells, consistent with a role for these proteins in modulating host cell vesicle trafficking. Interestingly, the ability of SetA to produce yeast phenotypes was dependent on a functional glycosyltransferase domain. We hypothesize that SetA may glycosylate a component of the host cell vesicle trafficking machinery during *L. pneumophila* infection [119].

Nino and colleagues further revealed, that the Dot/Icm system has the ability to spatially and temporally control the association of an effector with vacuoles containing *L. pneumophila* through activities mediated by other effector proteins [120]. It was determined that the association of PieA with vacuoles containing *L. pneumophila* requires modifications to the vacuole mediated by other Dot/Icm effectors. An example is PieA, an effector that can alter lysosome morphology and associates specifically with vacuoles that support *L. pneumophila* replication [120]. The Dot/Icm type-IVB secretion system plays an indisputably decisive role in allowing *L. pneumophila* to inhibit phagolysosome fusion. However, it was shown that LPS-rich outer membrane vesicles are also sufficient to inhibit phagosome–lysosome fusion by a mechanism that is correlated with developmentally regulated modifications of the pathogen's surface, but independent of type IV secretion [121].

Until recently the translocation signal for the type-IV secretion effectors was not known. Nagi and colleagues (2005) who investigated the mechanism of translocation of RalF [122] identified a 20-aa C-terminal region of the RalF protein as necessary and sufficient for translocation. In particular, a hydrophobic residue at the C-terminal -3 position is critical for secretion of RalF as substitution of the hydrophilic residues resulted in severe defects in translocation. Comparison with other Dot/Icm substrates showed that the majority contained a hydrophobic residue or a proline residue at the -3 or -4 positions, which supported the idea that these residues are critical for secretion using the type IV system. Based on these findings, Kubori and colleagues (2008) aligned the putative translocation signals of known Dot/Icm T4SS substrate proteins, to search for specific features regarding the occurrence of amino acids near the hydrophobic residues with which the sequences were aligned [123]. One of the most striking characteristics identified was that amino acids having very short side-chains (alanine, glycine, serine and threonine) are frequently found on residues at positions –8 to –2 (from the

hydrophobic residue). At the −2 position residue, the frequency of small amino acids (in ~80% of known substrates) is extremely high compared with that of all open reading frames (ORFs) from *L. pneumophila* (24%). Second, polar amino acids are clearly favored at residues −13 to +1 (in 65% of known substrates compared with 48% of all ORFs). This may reflect the solvent-exposed nature of the translocation signals. Establishment of a program to calculate 'similarity score to known Dot/Icm substrates' for any given protein from the frequency of occurrences of particular types of amino acid in the carboxy-terminal region spanning from −11 to +1 residues after alignment on the hydrophobic residue, led to the successful identification of 19 novel Dot/Icm substrate proteins [123].

6.3.3
Molecular Mimicry: the Main Virulence Strategy of *L. pneumophila*

A particular feature of *Legionella* is its dual host system which allows intracellular growth in protozoa and replication in human alveolar macrophages during infection. The capacity of pathogens like *Legionella* to infect eukaryotic cells is intimately linked to their ability to manipulate host cell functions to establish an intracellular niche for their replication. In 1980 Rowbotham described the ability of *Legionella* to multiply intracellularly in aquatic amoeba [124]. This observation has led to a new percept in microbiology: bacteria parasitize protozoa and can utilize the same process to infect humans. Indeed this early observation has recently been further substantiated when the first complete genome sequences of different *L. pneumophila* strains became available [95, 125, 126]. A particular feature of *L. pneumophila* that was identified by genome analysis was the presence of a large number and wide variety of proteins showing very strong similarities to eukaryotic proteins or encoding motifs known to be implicated in interactions with proteins which are present only or primarily in eukaryotes. Thus it is tempting to assume that the interaction of *L. pneumophila* with aquatic protozoa has generated this pool of virulence traits during evolution, which has also led to the ability of *Legionella* to infect human cells.

According to sequence analysis, the identified proteins were predicted to be involved in all the various stages of the *Legionella* life cycle, namely invasion, trafficking in the host cell, modulation of host cell functions and evasion from the host cell. Furthermore, recent analysis of the gene content of over 200 *L. pneumophila* strains by DNA–DNA hybridization showed that the eukaryotic-like genes identified during sequence analysis are conserved in all the strains tested with only a few exceptions [94]. These results underline their importance in the *L. pneumophila* life cycle and support the hypothesis that they are required for intracellular growth and thus play a role in virulence. The protein-motifs which are predominantly found in eukaryotes and which were identified in *L. pneumophila* genomes include ankyrin repeats, Sel-1 (TPR), Set domain, Sec7, serine threonine kinase domains (STPK), U-box and F-box motifs. Examples of eukaryotic-like proteins are two secreted apyrases, a sphingosine phosphate lyase and sphingosine kinase, eukaryotic-like glycoamylase, cytokinin oxidase, zinc metalloprotease and an RNA

binding precursor [95]. Recent functional studies confirm these predictions. The role of approximately 15 of the identified eukaryotic-like proteins or proteins containing a eukaryotic domain has meanwhile been investigated and their involvement in virulence and host cell modulation has been confirmed. Most of these proteins are also candidates for secretion by the Dot/Icm T4SS, as they must be translocated to the host cytoplasm to be able to affect the eukaryotic cell.

6.3.4 Implication of Eukaryotic-like Proteins in Virulence and Host Cell Modulation

6.3.4.1 Entry into and Blockade of Phagosomal–Lysosomal Fusion

After adhesion to a phagocytic cell, it is thought that *L. pneumophila* is subjected to host-driven phagocytosis. Once *L. pneumophila* has entered the eukaryotic host, it is able to modulate trafficking so that the *Legionella*-containing phagosome or *Legionella*-containing vacuole (LCV) is completely isolated from the host endocytic pathway and lysosome. Shortly after bacterial internalization, LCVs are found associated with endoplasmic reticulum-derived vesicles (for recent reviews see [127, 128]). After replication and depletion of nutrients the LCVs undergo maturation following a pathway similar to the autophagy pathway. The egress of bacteria following completion of replication is probably due to the formation of a second pore in addition to the Dot/Icm transporter pore, thus facilitating host lysis (for recent reviews see [127, 128]). To date it is only partly understood how *L. pneumophila* is able to subvert host functions to replicate inside eukaryotic cells and provoke pneumonia.

A eukaryotic-like protein in *L. pneumophila*, predicted to be an ecto-nucleoside triphosphate diphosphohydrolases (ecto-NTPDases) that share similarities with human CD39 and other eukaryotic ecto-NTPDases, has been shown to play a role in the uptake of *L. pneumophila* into the host cell. In humans, CD39 is located on the surface of endothelial cells and it controls extracellular levels of ATP by converting it into its diphosphate and monophosphate forms. In this way it plays a major role in maintaining vascular fluidity by regulating platelet aggregation [129]. CD39/NTPDases are found in a wide range of pathogens such as protozoan parasites, but their role in infection is poorly understood. One of the two predicted ecto-NTPDases in *L. pneumophila* is secreted into the host cell and is required for successful infection. The absence of this enzyme was not correlated with the ability to recruit the ER or avoidance of phago-lysosomal fusion but mainly to less efficient entry into the host cell [130]. Recently, it was shown that the enzyme catalyzed the hydrolysis of ATP and ADP and also of GTP and GDP but had only limited activity with CTP, CDP, UTP, and UDP. Furthermore, mutational analysis revealed that all five apyrase domains are necessary for infection following intratracheal inoculation of A/J mice [131].

The Dot/Icm-translocated proteins VipA, VipD, VipF are thought to participate in blocking lysosomal fusion. They have been identified in a yeast screen as the *L. pneumophila* proteins able to cause vacuolar missorting and to inhibit yeast lysosomal protein trafficking [115]. Two of these proteins (VipA and VipD) contain

eukaryotic-like domains. VipA contains a large coiled-coil region. These regions usually form highly versatile structures which are involved in protein–protein interactions commonly found in trafficking components such as soluble N-ethylmalemide-sensitive fusion attachment receptor proteins (SNARE) and early endosomal antigen 1 (EEA1). VipD is characterized by a patatin domain with strong homology to eukaryotic phospholipase A2 proteins. As suggested by its trafficking defect in yeast, VipD is thought to be involved in the intracellular infection process of *L. pneumophila* [115, 132]. Additional eukaryotic domain proteins shown to be implicated in modulating trafficking in the host cell are proteins that contain the eukaryotic Sel-1 domains. Sel-1 repeats represent a subfamily of tetratrico peptide repeats (TPRs) which are degenerate repeated motifs that form a scaffold in order to mediate protein–protein interactions. Three of the five Sel-1 domain-containing *L. pneumophila* proteins, LpnE, EnhC and LidL interact with the host cell to modulate early trafficking events that determine the fate of *Legionella* immediately after internalization and their growth within the host cell [133–136].

6.3.4.2 **Establishment of an ER-derived Replication Vacuole**

To promote fusion to ER membranes *L. pneumophila* recruits host factors such as Arf-1 and Rab-1 to the surface of the LCVs, these are important cell signaling proteins which are involved in the regulation of the ER-Golgi traffic. The *L. pneumophila* gene *ralF* encodes a protein with a Sec-7 domain. These domains are found in eukaryotes as components of Arf-specific guanine nucleotide exchange factors (GEFs). GEFs catalyze the nucleotide exchange of Arfs thereby converting them from an inactive (GDP-bound) to an active state (GTP-bound). Following secretion by T4SS, RalF recruits Arf-1 and then functions as an Arf-1-specific GEF [102]. The Dot/Icm translocated effector DrrA or SidM which is also able to interact with Rab1, has already been described above as it is not one of the eukaryotic-like effectors [106, 107].

The 20 ankyrin proteins in *L. pneumophila* are another interesting example of eukaryotic domain-containing proteins. The ankyrin domain is a 33-residue L-shaped motif containing two anti-parallel alpha-helices connected by a short loop [137]. The modular architecture and variable modular surfaces generated by the assembly of multiple compatible repeats render ankyrin proteins highly versatile in protein binding. This versatility and the multiple associated roles make the prediction of their function difficult. Ankyrin proteins are involved in cell signaling, cytoskeleton integrity and regulation, transcription and cell cycle regulation, inflammatory response and oncogenesis [138]. *L. pneumophila* single mutants for 11 of the 13 ankyrin proteins in *L. pneumophila* Philadelphia have been generated and analyzed. Two of these, known as AnkH and AnkJ, play a role in intracellular replication during infection of protozoan hosts [139]. Furthermore, the AnkX protein was shown to prevent microtubule-dependent vesicular transport which leads to the prevention of fusion of the LCV with late endosomes after infection of macrophages [140] It is not yet known whether the apparent redundancy of these ankyrin proteins or of other bacterial effectors masks a possible role in the

virulence of the remaining ankyrin proteins or whether these are not involved in protozoan host tropism.

6.3.4.3 Replication in the LCV and Egress from the Host

During bacterial replication unidentified ubiquitinated proteins are recruited to the LCV in a Dot/Icm-dependent manner [112]. Although the presence of these ubiquitinated proteins seems to be very important for bacterial replication the mechanism of their recruitment is unknown. Interestingly, the *L. pneumophila* genome encodes proteins containing domains with high similarity to F- and U-box domains of eukaryotic proteins [95]. F- and U-box domains are found in eukaryotic E3-ubiquitin ligases where they act by recognizing the targets of the ubiquitination process and leading them to proteasomal degradation. It has been shown that the *L. pneumophila* U-box containing effector, known as LubX, possesses *in vitro* ubiquitin ligase activity specific for the Cdc2-like kinase Clk1. While pharmacological inhibition of Clk1 inhibits bacterial replication, indicating its role in intracellular replication of *L. pneumophila*, neither the replication nor any step in the intracellular cycle was impaired in a *lubX* mutant [123].

After completion of intracellular replication, bacteria must exit the exhausted host cell in order to infect a new one. The egress process in not well understood but the formation of an egress pore has been hypothesized [141]. Two Dot/Icm effectors have been shown to be implicated in an active but non-lytic egress of *L. pneumophila* from protozoan but not mammalian cells. These two effectors are LepA and LepB: both have weak homology to eukaryotic SNAREs. SNAREs are protein receptors that mediate vesicle-membrane fusions [142]. LepB also has Rab-GAP activity which is involved in the formation of LCVs, but it may also contain other functional domains involved in the escape of *L. pneumophila* from the host cells.

6.3.5 Evolution of the Eukaryotic-like Proteins

How did these eukaryotic-like proteins and protein domains which are clearly virulence factors used by *L. pneumophila* to subvert host functions, evolve? Two hypotheses may explain the presence of these proteins: horizontal gene transfer (HGT) and/or convergent evolution. The hypothesis of HGT is supported by the fact that most of these genes show a G+C bias as compared to other *L. pneumophila* genes [118]. In addition, *L. pneumophila* is naturally competent [143, 144], a characteristic that could explain the mechanism which facilitates the acquisition of foreign DNA. Furthermore, it has been demonstrated that *L. pneumophila* is able to transfer chromosomal DNA horizontally to other *L. pneumophila* cells [145] and that this bacterium is able to take up DNA from the environment [144]. It has been hypothesized that *ralF* was the first gene to have been acquired by *L. pneumophila* from eukaryotes by HGT. RalF carries a eukaryotic Sec 7 domain and is probably derived from a eukaryotic host [102]. However, in contrast to DNA exchange between closely related bacteria that are likely to be successful because of compatible methods of

genetic exchange, transfers between distantly related taxa are much less likely to succeed as the foreign DNA has to integrate into the genome via illegitimate rather than homologous recombination [146]. However, if organisms in the environment are subjected to a constant 'rain' of DNA, then these rare processes can take place and will be fixed in a lineage particularly if they confer a selective advantage on the recipient organism [147]. The interaction between *Legionella* and protozoa may have provided many opportunities for the incorporation of DNA from eukaryotes during the course of evolution. In fact, horizontal transfer from bacteria to amoeba has been already described as there appear to be 96 cases of relatively recent prokaryote-to-eukaryote HGT in the *Ecantamoeba histolytica* genome, and 18 potential instances of HGT in the *Dictyostelium discoideum* genome [148, 149].

Another way to explain the presence of these eukaryotic-like proteins and protein domains is convergent evolution. It might be suggested that this process could have taken place in the case of proteins in which only one portion of the sequence is functionally important; so few changes in the sequence would lead to evolutionary convergence of domain architectures. This might be the case for example for eukaryotic domains, since these are restricted to partial segments of the amino acid sequence. However, [150] it has been concluded from the analysis of the evolution of domain architectures across 62 genomes of known phylogeny including all kingdoms of life, that convergent evolution is rare. In any case, the two possibilities, horizontal transfer and convergent evolution are not exclusive; both can have taken place depending on the protein. Only future studies combining phylogenetic and structural information for each of these proteins, together with access to more completed protozoan genome sequences, will help to reveal the origin of each eukaryotic-like gene.

6.3.6
Legionella Genomes

To date, the complete genome sequences of four strains of *L. pneumophila* serogroup Sg1 have been published: strains Paris, Lens, Philadelphia and Corby [95, 125, 126]. The genomes of these strains are composed of a single circular chromosome of 3.34 Mb (strain Lens) to 3.57 Mb (strain Corby) in size. A single circular plasmid has been detected in the strains Lens and Paris. These genomes show substantial homogeneity with regard to GC content (~ 39%), coding percentage and average length of the coding sequences. The particular features of the *Legionella* genomes as deduced from sequence analyses are: (i) high genome plasticity as many pathogenicity islands and mobility genes have been discovered, (ii) high genetic diversity, as 7.5 to 10.5% of the genes of each strain are specific. This genome diversity is further underlined by a recent study comparing the gene content of over 200 *L. pneumophila* strains. Except for known and putative virulence factors, which are highly conserved among the strains investigated, *L. pneumophila* is a genetically diverse species [94]. The most intriguing feature of the *L. pneumophila* genomes, discovered by genome sequencing and genome analysis, is the presence of (iii) a large number and wide variety of eukaryotic-like proteins

or eukaryotic protein domains as described above [95, 118, 151]. The sequencing of several additional isolates of *L. pneumophila* and of other species of *Legionella* is currently under way and is nearing completion. This will lead to further insight into the particular virulence strategies of *L. pneumophila* and to the understanding of the evolution of these genomes.

6.3.7 Host response to *Legionella* infection

Legionella pneumophila is a model organism used to investigate the initiation of innate immunity to intracellular bacterial pathogens. Its ability to create a specialized vacuole in which it replicates represents a unique challenge for the host immune system. Although *L. pneumophila* causes legionellosis, most infections caused by *L. pneumophila* are cleared by the host immune response, which is why most infections are asymptomatic. In mice, replication of *L. pneumophila* in the lung is usually suppressed after 2 days of infection [152]. Soon after infection by *L. pneumophila*, inflammatory cytokines such as interleukine-12 (IL-12) and IL-18 which play a role in activating natural killer (NK) and NK T cells to produce interferon-gamma (IFN-γ) and tumor necrosis factor alpha (TNF-α) are produced; [152–154] neutrophils are also recruited at this stage [155] to restrict the initial replication of *L. pneumophila* cells. Two pathways which are activated by *L. pneumophila* infection have been studied in greater detail: the clearance of *L. pneumophila* by a MyD88 response and the Naip-5-mediated innate immune response.

6.3.7.1 The MyD88 Response is Important for the Control of *L. pneumophila* Infection

It has been shown in a mouse model that IFN-γ produced by natural killer (NK) cells after infection is a key factor in the early clearance of *L. pneumophila* in the lungs [152, 156]. MyD88, a major adaptor protein implicated in Toll-like receptor (TLR) signaling, was implicated in this process and TLR2, but not TLR4, was described as one of the pattern recognition receptors involved in initiating this MyD88-dependent innate immune response [152, 156]. Indeed, MyD88 was shown to be indispensable to IL12p40, IL6 and TNF-α cytokine production by *L. pneumophila*-infected macrophages [152]. Furthermore, it is known that IL12 is a potent stimulator of IFN-γ production by NK-cells [157] and is critical for immunoregulation against *L. pneumophila* [155, 158]. Thus it is thought that the production of IL-12 and other pro-inflammatory cytokines following the recognition of *L. pneumophila* by MyD88 might affect IFN-γ production and thus interfere with the activation of macrophages which are capable of restricting the intracellular growth of *L. pneumophila* [152]. Recently it was shown that the MyD88-dependent innate immune responses induced by *L. pneumophila* involved both TLR-dependent responses and also IL-18R-dependent production of IFN-γ by natural killer cells, and that these MyD88-dependent pathways can function independently to provide host protection against this intracellular pathogen [159].

6.3.7.2 Naip-5-Dependent Immune Response to Cytosolic Flagellin

Studies of the genetic basis and mechanisms involved in mouse susceptibility or resistance to *L. pneumophila* led to the determination of another pathway in the innate immunity process which controls *L. pneumophila* infection. Several studies have implicated the host protein Naip5 (Birc1e) in the resistance to *L. pneumophila* [160, 161]. More recently, it was found that the Naip5-dependent pro-inflammatory macrophage response is triggered by flagellin and results in the induction of IL-1β secretion [162–164]. This response requires the pore-forming action of the Dot/Icm T4SS system so that flagellin can diffuse into the cytosol where it is recognized by Naip5; caspase-1 is also required for the successful secretion of IL-1β [162]. The proteins Asc and Ipaf are known adaptors that regulate the activation of caspase-1. Their implication in the Naip-5-dependent response has been investigated and it was shown that Naip-5 and Ipaf interact to restrict *L. pneumophila* growth in a caspase-1-dependent manner although independently of IL-1β secretion, while Naip-5, Ipaf and Asc are essential for IL-1β secretion [164]. It was proposed that upon activation, macrophages respond to infection by several processes, for example release of inflammatory cytokines, degradation of intracellular bacteria and self-destruction.

6.3.7.3 Dictyostelium Transcriptional Host Cell Response upon Infection

The social amoeba *Dictyostelium discoideum* is a well-defined model organism used in the study of *L. pneumophila* infections and its use has led to a better understanding of the complex host–pathogen cross talk [165, 166]. The similarities between *Dictyostelium* and mammalian cells include cytoskeletal proteins, membrane trafficking, endocytic transit and sorting events. Various studies showed that the growth kinetics of pathogenic and non-pathogenic *Legionella* species as well as *Legionella* mutants in *Dictyostelium* correlate with infections of human macrophages such as cell lines or protozoa [167–169].

Recent studies have used microarrays carrying half of the *D. dicoideum* genes to study the host cell response to *Legionella* infection [170]. Analysis of the differentially regulated genes showed that following *L. pneumophila* infection the host ribosome constituents, protein biosynthesis, hydrolases, lysozyme activity, lipid-modifying enzymes and a number of small calcium-binding proteins are all down-regulated. In contrast, genes encoding enzymes involved in tRNA metabolism and modification, glucose homeostasis, the metabolism of amino acids belonging to the glutamine and pyruvate family, nucleotide biosynthesis as well as cytoskeletal proteins were enriched among the up-regulated genes. Apart from a stress response which had already been described in the context of infection genes which have a clear link to pathogenesis, the host genes were differentially regulated. This is the case for the genes encoding RtoA, ARF1 and β′-COP. $RtoA^-$ cells (ratioA mutant) show abnormalities in vesicle fusion in endo- and exocytosis, while the ADP-ribosylation factor ARF1 and the coatomer protein β′-COP regulate and direct vesicular trafficking from the early secretory pathway. Further factors that could influence the fate of the phagosome in the host are certain fatty acid-modifying

enzymes and PIP-6 kinases. Furthermore, the upregulation of transcripts belonging to the ubiquitination machinery of *D. discoideum* after infection with *Legionella* wild type as compared to the *dotA* mutant was also observed [170]. It has been shown that *L. pneumophila* secretes via the Dot/Icm system, proteins that might interfere with the ubiquitination machinery of the host which may play a role in this process [123, 139]. Taken together, *Legionella* obviously exploits host cell functions such as the ubiquitination machinery and induces the downregulation of the host-specific metabolism and degradative enzymes and on the other hand induces the upregulation of activities that produce nutrients suitable for the pathogen.

6.4 Conclusions

Legionella and Pneumococci are quite different types of bacteria: the former is a Gram-negative rod and an intracellular pathogen while the latter is a Gram-positive coccus which has not as yet been found as an intracellular pathogen. Their virulence strategies are also quite different. The virulence of *Legionella* is mainly determined by secreted substrates which allow this organism to mimic host cell functions and many of these secreted substrates seem to have been acquired by horizontal gene transfer or convergent evolution. In pneumococci virulence is based mainly on a large number of different virulence factors including surface-anchored proteins and the capsule. *Legionella* are environmental bacteria that are accidental pathogens for humans while pneumococci are part of the normal flora of the nasopharynx of humans and are not found in the environment. However, both bacteria cause pneumonia with a number of similar symptoms. Furthermore, the host defense mechanisms seem to be similar. However, it is not as yet known whether neutrophils also respond with NET formation to fight *Legionella* infection as they do for infection with *S. pneumoniae*. It is likely that this question will be investigated further in future studies. The development of many new high throughput techniques for comparative and functional genomics will also help to better understand and fight diseases and with the accumulation of more data systems biological approaches might lead to new answers.

Acknowledgment

Financial support was obtained from the Institut Pasteur, the Centre National de la Recherche (CNRS), the Network of Excellence "Europathogenomics" LSHB-CT-2005-512061, the DIM Maladie Infectieuses, the INTRAPTH project MEST-CT-2005-020715, Torsten and Ragnar Söderbergs foundation, and the Swedish Research Council.

References

1 Bartlett, J.G., Dowell, S.F., Mandell, L.A., File, T.M., Jr., Musher, D.M., and Fine, M.J. (2000) *Clin. Infect. Dis.*, **31**, 347–382.

2 Sharpe, B.A., and Flanders, S.A. (2006) *J. Hosp. Med.*, **1**, 177–190.

3 Tomasz, A. (1997) *Clin. Infect. Dis.*, **24** (Suppl. 1), S85–S88.

4 Goldbart, A.D., Leibovitz, E., Porat, N., Givon-Lavi, N., Drukmann, I., Tal, A., and Greenberg, D. (2009) *Scand. J. Infect. Dis.*, **41** (3), 182–187.

5 Brueggemann, A.B., Peto, T.E., Crook, D.W., Butler, J.C., Kristinsson, K.G., and Spratt, B.G. (2004) *J. Infect. Dis.*, **190**, 1203–1211.

6 Sandgren, A., Sjostrom, K., Olsson-Liljequist, B., Christensson, B., Samuelsson, A., Kronvall, G., and Henriques Normark, B. (2004) *J. Infect. Dis.*, **189**, 785–796.

7 Sjostrom, K., Spindler, C., Ortqvist, A., Kalin, M., Sandgren, A., Kuhlmann-Berenzon, S., and Henriques-Normark, B. (2006) *Clin. Infect. Dis.*, **42**, 451–459.

8 Hoskins, J., Alborn, W.E., Jr., Arnold, J., Blaszczak, L.C., Burgett, S., DeHoff, B.S., Estrem, S.T., Fritz, L., Fu, D.J., Fuller, W., Geringer, C., Gilmour, R., Glass, J.S., Khoja, H., Kraft, A.R., Lagace, R.E., LeBlanc, D.J., Lee, L.N., Lefkowitz, E.J., Lu, J., Matsushima, P., McAhren, S.M., McHenney, M., McLeaster, K., Mundy, C.W., Nicas, T.I., Norris, F.H., O'Gara, M., Peery, R.B., Robertson, G.T., Rockey, P., Sun, P.M., Winkler, M.E., Yang, Y., Young-Bellido, M., Zhao, G., Zook, C.A., Baltz, R.H., Jaskunas, S.R., Rosteck, P.R., Jr., Skatrud, P.L., and Glass, J.I. (2001) *J. Bacteriol.*, **183**, 5709–5717.

9 Tettelin, H., Nelson, K.E., Paulsen, I.T., Eisen, J.A., Read, T.D., Peterson, S., Heidelberg, J., DeBoy, R.T., Haft, D.H., Dodson, R.J., Durkin, A.S., Gwinn, M., Kolonay, J.F., Nelson, W.C., Peterson, J.D., Umayam, L.A., White, O., Salzberg, S.L., Lewis, M.R., Radune, D., Holtzapple, E., Khouri, H., Wolf, A.M., Utterback, T.R., Hansen, C.L., McDonald, L.A., Feldblyum, T.V., Angiuoli, S., Dickinson, T., Hickey, E.K., Holt, I.E., Loftus, B.J., Yang, F., Smith, H.O., Venter, J.C., Dougherty, B.A., Morrison, D.A., Hollingshead, S.K., and Fraser, C.M. (2001) *Science*, **293**, 498–506.

10 Blomberg, B., Dagerhamn, J., Dahlberg, S., Browall, S., Fernebro, J., Albiger, B., Morfeldt, E., Normark, S., and Henriques-Normark, B. (2009) *J. Infect. Dis.*, **199** (7), 1032–1042.

11 Chiavolini, D., Pozzi, G., and Ricci, S. (2008) *Clin. Microbiol. Rev.*, **21**, 666–685.

12 van Ginkel, F.W., McGhee, J.R., Watt, J.M., Campos-Torres, A., Parish, L.A., and Briles, D.E. (2003) *Proc. Natl. Acad. Sci. USA*, **100** (24), 14363–14367.

13 Sandgren, A., Albiger, B., Orihuela, C.J., Tuomanen, E., Normark, S., and Henriques-Normark, B. (2005) *J. Infect. Dis.*, **192**, 791–800.

14 Steinmoen, H., Knutsen, E., and Havarstein, L.S. (2002) *Proc. Natl Acad. Sci. USA*, **99**, 7681–7686.

15 Johnsborg, O., Kristiansen, P.E., Blomqvist, T., and Havarstein, L.S. (2006) *J. Bacteriol.*, **188**, 1744–1749.

16 Martin, B., Quentin, Y., Fichant, G., and Claverys, J.P. (2006) *Trends Microbiol.*, **14**, 339–345.

17 Claverys, J.P., Martin, B., and Havarstein, L.S. (2007) *Mol. Microbiol.*, **64**, 1423–1433.

18 Claverys, J.P., and Havarstein, L.S. (2007) *Nat. Rev. Microbiol.*, **5**, 219–229.

19 Kadioglu, A., Weiser, J.N., Paton, J.C., and Andrew, P.W. (2008) *Nat. Rev. Microbiol.*, **6**, 288–301.

20 Lysenko, E.S., Ratner, A.J., Nelson, A.L., and Weiser, J.N. (2005) *PLoS Pathog.*, **1**, e1.

21 Ratner, A.J., Aguilar, J.L., Shchepetov, M., Lysenko, E.S., and Weiser, J.N. (2007) *Cell. Microbiol.*, **9**, 1343–1351.

22 Bogaert, D., van Belkum, A., Sluijter, M., Luijendijk, A., de Groot, R., Rümke, H.C., Verbrugh, H.A., and Hermans, P.W. (2004) *Lancet*, **363** (9424), 1871–1872.

23 Regev-Yochay, G., Dagan, R., Raz, H., Carmeli, Y., Shainberg, B., Derazne, E.,

Rahav, G., and Rubinstein, E. (2004) *JAMA*, **292**(6), 716–720.
24 Melles, D.C., Bogaert, D., Gorkink, R.F., Peeters, J.K., Moorhouse, M.J., Ott, A., van Leeuwen, W.B., Simons, G., Verbrugh, H.A., Hermans, P.W., and van Belkum, A. (2007) *Microbiology*, **153**, 686–692.
25 Veenhoven, R., Bogaert, D., Uiterwaal, C., Brouwer, C., Kiezebrink, H., Bruin, J., Ijzerman, E., Hermans, P., de Groot, R., Zegers, B., Kuis, W., Rijkers, G., Schilder, A., and Sanders, E. (2003) *Lancet*, **361**, 2189–2195.
26 Herz, A.M., Greenhow, T.L., Alcantara, J., Hansen, J., Baxter, R.P., Black, S.B., and Shinefield, H.R. (2006) *Pediatr. Infect. Dis. J.*, **25**, 293–300.
27 Dawid, S., Roche, A.M., and Weiser, J.N. (2007) *Infect. Immun.*, **75**, 443–451.
28 Dawid, S., Sebert, M.E., and Weiser, J.N. (2009) *J. Bacteriol.*, **191** (5), 1508–1518.
29 Munoz-Elias, E.J., Marcano, J., and Camilli, A. (2008) *Infect. Immun.*, **76**, 5049–5061.
30 Oggioni, M.R., Trappetti, C., Kadioglu, A., Cassone, M., Iannelli, F., Ricci, S., Andrew, P.W., and Pozzi, G. (2006) *Mol. Microbiol.*, **61**, 1196–1210.
31 McCullers, J.A. (2006) *Clin. Microbiol. Rev.*, **19**, 571–582.
32 Madhi, S.A., and Klugman, K.P. (2004) *Nat. Med.*, **10**, 811–813.
33 Peltola, V.T., and McCullers, J.A. (2004) *Pediatr. Infect. Dis. J.*, **23** (1 Suppl), S87–97.
34 Sun, K., and Metzger, D.W. (2008) *Nat. Med.*, **14**, 558–564.
35 Mavroidi, A., Aanensen, D.M., Godoy, D., Skovsted, I.C., Kaltoft, M.S., Reeves, P.R., Bentley, S.D., and Spratt, B.G. (2007) *J. Bacteriol.*, **189**, 7841–7855.
36 Hammerschmidt, S., Wolff, S., Hocke, A., Rosseau, S., Müller, E., and Rohde, M. (2005) *Infect. Immun.*, **73** (8), 4653–4667.
37 Hammerschmidt, S. (2006) *Curr. Opin. Microbiol.*, **9**, 12–20.
38 Jedrzejas, M.J. (2006) *Clin. Chim. Acta*, **367**, 1–10.
39 Ren, B., McCrory, M.A., Pass, C., Bullard, D.C., Ballantyne, C.M., Xu, Y., Briles, D.E., and Szalai, A.J. (2004) *J. Immunol.*, **173**, 7506–7512.
40 Darrieux, M., Moreno, A.T., Ferreira, D.M., Pimenta, F.C., de Andrade, A.L., Lopes, A.P., Leite, L.C., and Miyaji, E.N. (2008) *J. Med. Microbiol.*, **57**, 273–278.
41 Rosenow, C., Ryan, P., Weiser, J.N., Johnson, S., Fontan, P., Ortqvist, A., and Masure, H.R. (1997) *Mol. Microbiol.*, **25**, 819–829.
42 Bergmann, S., and Hammerschmidt, S. (2006) *Microbiology*, **152**, 295–303.
43 Kerr, A.R., Paterson, G.K., McCluskey, J., Iannelli, F., Oggioni, M.R., Pozzi, G., and Mitchell, T.J. (2006) *Infect. Immun.*, **74**, 5319–5324.
44 Orihuela, C.J., Gao, G., Francis, K.P., Yu, J., and Tuomanen, E.I. (2004) *J. Infect. Dis.*, **190**, 1661–1669.
45 Lopez, R., and Garcia, E. (2004) *FEMS Microbiol. Rev.*, **28**, 553–580.
46 Gosink, K.K., Mann, E.R., Guglielmo, C., Tuomanen, E.I., and Masure, H.R. (2000) *Infect. Immun.*, **68**, 5690–5695.
47 Dintilhac, A., Alloing, G., Granadel, C., and Claverys, J.P. (1997) *Mol. Microbiol.*, **25**, 727–739.
48 Orihuela, C.J., Radin, J.N., Sublett, J.E., Gao, G., Kaushal, D., and Tuomanen, E.I. (2004) *Infect. Immun.*, **72**, 5582–5596.
49 Rajam, G., Anderton, J.M., Carlone, G.M., Sampson, J.S., and Ades, E.W. (2008) *Crit. Rev. Microbiol.*, **34**, 131–142.
50 Barocchi, M.A., Ries, J., Zogaj, X., Hemsley, C., Albiger, B., Kanth, A., Dahlberg, S., Fernebro, J., Moschioni, M., Masignani, V., Hultenby, K., Taddei, A.R., Beiter, K., Wartha, F., von Euler, A., Covacci, A., Holden, D.W., Normark, S., Rappuoli, R., and Henriques-Normark, B. (2006) *Proc. Natl Acad. Sci. USA*, **103**, 2857–2862.
51 Falker, S., Nelson, A.L., Morfeldt, E., Jonas, K., Hultenby, K., Ries, J., Melefors, O., Normark, S., and Henriques-Normark, B. (2008) *Mol. Microbiol.*, **70**, 595–607.
52 Hilleringmann, M., Giusti, F., Baudner, B.C., Masignani, V., Covacci, A., Rappuoli, R., Bavocchi, M.A., and Ferlenghi, I. (2008) *PLoS Pathog.*, **4** (3), e1000026.
53 Neiers, F., Madhurantakam, G., Fälker, S., Manzano, C., Dessen, A., Normark,

S., Henriques-Normark, B., and Achow, A. (2009) *J. Mol. Biol.*, **393** (3), 704–716.

54 Manzano, G., Contreras-Martel, C., El Mortaji, L., Jzoré, T., Fenel, D., Vernet, T., Schoehn, G., Di Guilmi, A.M., and Dessen, A. (2008) *Structure*, **16** (12), 1838–1848.

55 Nelson, A.L., Ries, J., Bagnoli, F., Dahlberg, S., Falker, S., Rounioja, S., Tschop, J., Morfeldt, E., Ferlenghi, I., Hilleringmann, M., Holden, D.W., Rappuoli, R., Normark, S., Barocchi, M.A., and Henriques-Normark, B. (2007) *Mol. Microbiol.*, **66**, 329–340.

56 Sjostrom, K., Blomberg, C., Fernebro, J., Dagerhamn, J., Morfeldt, E., Barocchi, M.A., Browall, S., Moschioni, M., Andersson, M., Henriques, F., Albiger, B., Rappuoli, R., Normark, S., and Henriques-Normark, B. (2007) *Proc. Natl Acad. Sci. USA*, **104**, 12907–12912.

57 Bagnoli, F., Moschioni, M., Donati, C., Dimitrovska, V., Ferlenghi, I., Facciotti, C., Muzzi, A., Giusti, F., Emolo, C., Sinisi, A., Hilleringmann, M., Pansegrau, W., Censini, S., Rappuoli, R., Covacci, A., Masignani, V., and Barocchi, M.A. (2008) *J. Bacteriol.*, **190**, 5480–5492.

58 Weiser, J.N., Bae, D., Fasching, C., Scamurra, R.W., Ratner, A.J., and Janoff, E.N. (2003) *Proc. Natl Acad. Sci. USA*, **100**, 4215–4220.

59 Oggioni, M.R., Memmi, G., Maggi, T., Chiavolini, D., Iannelli, F., and Pozzi, G. (2003) *Mol. Microbiol.*, **49**, 795–805.

60 Rose, L., Shivshankar, P., Hinojosa, E., Rodriguez, A., Sanchez, C.J., and Orihuela, C.J. (2008) *J. Infect. Dis.*, **198**, 375–383.

61 Kirkham, L.A., Jefferies, J.M., Kerr, A.R., Jing, Y., Clarke, S.C., Smith, A., and Mitchell, T.J. (2006) *J. Clin. Microbiol.*, **44**, 151–159.

62 Jefferies, J.M., Johnston, C.H., Kirkham, L.A., Cowan, G.J., Ross, K.S., Smith, A., Clarke, S.C., Brueggemann, A.B., George, R.C., Pichon, B., Pluschke, G., Pfluger, V., and Mitchell, T.J. (2007) *J. Infect. Dis.*, **196**, 936–944.

63 Marriott, H.M., Mitchell, T.J., and Dockrell, D.H. (2008) *Curr. Mol. Med.*, **8**, 497–509.

64 Marriott, H.M., and Dockrell, D.H. (2006) *Int. J. Biochem. Cell Biol.*, **38**, 1848–1854.

65 Dockrell, D.H., Marriott, H.M., Prince, L.R., Ridger, V.C., Ince, P.G., Hellewell, P.G., and Whyte, M.K. (2003) *J. Immunol.*, **171**, 5380–5388.

66 Srivastava, A., Henneke, P., Visintin, A., Morse, S.C., Martin, V., Watkins, C., Paton, J.C., Wessels, M.R., Golenbock, D.T., and Malley, R. (2005) *Infect. Immun.*, **73**, 6479–6487.

67 Regev-Yochay, G., Trzcinski, K., Thompson, C.M., Lipsitch, M., and Malley, R. (2007) *J. Bacteriol.*, **189**, 6532–6539.

68 Brinkmann, V., Reichard, U., Goosmann, C., Fauler, B., Uhlemann, Y., Weiss, D.S., Weinrauch, Y., and Zychlinsky, A. (2004) *Science*, **303**, 1532–1535.

69 Albiger, B., Dahlberg, S., Henriques-Normark, B., and Normark, S. (2007) *J. Intern. Med.*, **261**, 511–528.

70 Albiger, B., Sandgren, A., Katsuragi, H., Meyer-Hoffert, U., Beiter, K., Wartha, F., Hornef, M., Normark, S., and Normark, B.H. (2005) *Cell. Microbiol.*, **7**, 1603–1615.

71 Malley, R., Henneke, P., Morse, S.C., Cieslewicz, M.J., Lipsitch, M., Thompson, C.M., Kurt-Jones, E., Paton, J.C., Wessels, M.R., and Golenbock, D.T. (2003) *Proc. Natl Acad. Sci. USA*, **100**, 1966–1971.

72 Albiger, B., Dahlberg, S., Sandgren, A., Wartha, F., Beiter, K., Katsuragi, H., Akira, S., Normark, S., and Henriques-Normark, B. (2007) *Cell. Microbiol.*, **9**, 633–644.

73 Arredouani, M., Yang, Z., Ning, Y., Qin, G., Soininen, R., Tryggvason, K., and Kobzik, L. (2004) *J. Exp. Med.*, **200**, 267–272.

74 Beiter, K., Wartha, F., Albiger, B., Normark, S., Zychlinsky, A., and Henriques-Normark, B. (2006) *Curr. Biol.*, **16**, 401–407.

75 Wartha, F., Beiter, K., Albiger, B., Fernebro, J., Zychlinsky, A., Normark,

S., and Henriques-Normark, B. (2007) *Cell. Microbiol.*, **9**, 1162–1171.
76 Beiter, K., Wartha, F., Hurwitz, R., Normark, S., Zychlinsky, A., and Henriques-Normark, B. (2008) *Infect. Immun.*, **76**, 3710–3716.
77 Hiller, N.L., Janto, B., Hogg, J.S., Boissy, R., Yu, S., Powell, E., Keefe, R., Ehrlich, N.E., Shen, K., Hayes, J., Barbadora, K., Klimke, W., Dernovoy, D., Tatusova, T., Parkhill, J., Bentley, S.D., Post, J.C., Ehrlich, G.D., and Hu, F.Z. (2007) *J. Bacteriol.*, **189**, 8186–8195.
78 Bambini, S., and Rappuoli, R. (2009) *Drug Discov. Today*, **14** (5–6), 252–260.
79 Diederen, B.M. (2008) *J. Infect.*, **56**, 1–12.
80 McDade, J.E., Shepard, C.C., Fraser, D.W., Tsai, T.R., Redus, M.A., and Dowdle, W.R. (1977) *N. Engl. J. Med.*, **297**, 1197–1203.
81 Marrie, T.J. (2008) *Legionella Pneumophila: Pathogenesis and Immunity* (eds P. Hoffmann, H. Friedman, and M. Bendinelli), Springer, New York, pp. 133–150.
82 Marston, B.J., Lipman, H.B., and Breiman, R.F. (1994) *Arch. Intern. Med.*, **154**, 2417–2422.
83 Joseph, C., and Ricketts, K. (2008) *Legionella: Molecular Microbiology* (eds K. Heuner and M. Swanson), Caister Academic Press, Norfolk, UK, pp. 35–54.
84 Rusin, P.A., Rose, J.B., Haas, C.N., and Gerba, C.P. (1997) *Rev. Environ. Contam. Toxicol.*, **152**, 57–83.
85 Benin, A.L., Benson, R.F., Arnold, K.E., Fiore, A.E., Cook, P.G., Williams, L.K., Fields, B., and Besser, R.E. (2002) *J. Infect. Dis.*, **185**, 237–243.
86 Fields, B.S., Benson, R.F., and Besser, R.E. (2002) *Clin. Microbiol. Rev.*, **15**, 506–526.
87 Yu, V.L., Plouffe, J.F., Pastoris, M.C., Stout, J.E., Schousboe, M., Widmer, A., Summersgill, J., File, T., Heath, C.M., Paterson, D.L., and Chereshsky, A. (2002) *J. Infect. Dis.*, **186**, 127–128.
88 Doleans, A., Aurell, H., Reyrolle, M., Lina, G., Freney, J., Vandenesch, F., Etienne, J., and Jarraud, S. (2004) *J. Clin. Microbiol.*, **42**, 458–460.
89 Harrison, T.G., Afshar, B., Doshi, N., Fry, N.K., and Lee, J.V. (2009) *Eur. J. Clin. Microbiol. Infect. Dis.*, **28** (7), 781–791.
90 Muder, R.R., and Yu, V.L. (2002) *Clin. Infect. Dis.*, **35**, 990–998.
91 Steele, T.W., Moore, C.V., and Sangster, N. (1990) *Appl. Environ. Microbiol.*, **56**, 2984–2988.
92 Aurell, H., Farge, P., Meugnier, H., Gouy, M., Forey, F., Lina, G., Vandenesch, F., Etienne, J., and Jarraud, S. (2005) *Appl. Environ. Microbiol.*, **71**, 282–289.
93 Bumbaugh, A.C., McGraw, E.A., Page, K.L., Selander, R.K., and Whittam, T.S. (2002) *Curr. Microbiol.*, **44**, 314–322.
94 Cazalet, C., Jarraud, S., Ghavi-Helm, Y., Kunst, F., Glaser, P., Etienne, J., and Buchrieser, C. (2008) *Genome Res.*, **18**, 431–441.
95 Cazalet, C., Rusniok, C., Bruggemann, H., Zidane, N., Magnier, A., Ma, L., Tichit, M., Jarraud, S., Bouchier, C., Vandenesch, F., Kunst, F., Etienne, J., Glaser, P., and Buchrieser, C. (2004) *Nat. Genet.*, **36**, 1165–1173.
96 Amemura-Maekawa, J., Kura, F., Chang, B., and Watanabe, H. (2005) *Microbiol. Immunol.*, **49**, 1027–1033.
97 Aurell, H., Etienne, J., Forey, F., Reyrolle, M., Girardo, P., Farge, P., Decludt, B., Vandenesch, F., and Jarraud, S. (2003) *J. Clin. Microbiol.*, **41**, 3320–3322.
98 Ginevra, C., Forey, F., Campese, C., Reyrolle, M., Che, D., Etienne, J., and Jarraud, S. (2008) *Emerg. Infect. Dis.*, **14**, 673–675.
99 Sexton, J.A., and Vogel, J.P. (2002) *Traffic*, **3**, 178–185.
100 Ensminger, A.W., and Isberg, R.R. (2009) *Curr. Opin. Microbiol.*, **12** (1), 67–73.
101 Ninio, S., and Roy, C.R. (2007) *Trends Microbiol.*, **15**, 372–380.
102 Nagai, H., Kagan, J.C., Zhu, X., Kahn, R.A., and Roy, C.R. (2002) *Science*, **295**, 679–682.
103 Conover, G.M., Derre, I., Vogel, J.P., and Isberg, R.R. (2003) *Mol. Microbiol.*, **48**, 305–321.

104 Chen, J., Reyes, M., Clarke, M., and Shuman, H.A. (2007) *Cell. Microbiol.*, **9**, 1660–1671.

105 Luo, Z.Q., and Isberg, R.R. (2004) *Proc. Natl Acad. Sci. USA*, **101**, 841–846.

106 Machner, M.P., and Isberg, R.R. (2006) *Dev. Cell*, **11**, 47–56.

107 Murata, T., Delprato, A., Ingmundson, A., Toomre, D.K., Lambright, D.G., and Roy, C.R. (2006) *Nat. Cell Biol.*, **8**, 971–977.

108 Ingmundson, A., Delprato, A., Lambright, D.G., and Roy, C.R. (2007) *Nature*, **450**, 365–369.

109 Weber, S.S., Ragaz, C., Reus, K., Nyfeler, Y., and Hilbi, H. (2006) *PLoS Pathog.*, **2**, e46.

110 Brombacher, E., Urwyler, S., Ragaz, C., Weber, S.S., Kami, K., Overduin, M., and Hilbi, H. (2008) *J. Biol. Chem.*, **284** (8), 4846–4856.

111 Banga, S., Gao, P., Shen, X., Fiscus, V., Zong, W.X., Chen, L., and Luo, Z.Q. (2007) *Proc. Natl Acad. Sci. USA*, **104**, 5121–5126.

112 Dorer, M.S., Kirton, D., Bader, J.S., and Isberg, R.R. (2006) *PLoS Pathog.*, **2**, e34.

113 Lesser, C.F., and Miller, S.I. (2001) *EMBO J.*, **20**, 1840–1849.

114 Slagowski, N.L., Kramer, R.W., Morrison, M.F., LaBaer, J., and Lesser, C.F. (2008) *PLoS Pathog.*, **4**, e9.

115 Shohdy, N., Efe, J.A., Emr, S.D., and Shuman, H.A. (2005) *Proc. Natl Acad. Sci. USA*, **102**, 4866–4871.

116 Campodonico, E.M., Chesnel, L., and Roy, C.R. (2005) *Mol. Microbiol.*, **56**, 918–933.

117 de Felipe, K.S., Glover, R.T., Charpentier, X., Anderson, O.R., Reyes, M., Pericone, C.D., and Shuman, H.A. (2008) *PLoS Pathog.*, **4**, e1000117.

118 de Felipe, K.S., Pampou, S., Jovanovic, O.S., Pericone, C.D., Ye, S.F., Kalachikov, S., and Shuman, H.A. (2005) *J. Bacteriol.*, **187**, 7716–7726.

119 Heidtman, M., Chen, E.J., Moy, M.Y., and Isberg, R.R. (2009) *Cell. Microbiol.*, **11**, 230–248.

120 Ninio, S., Celli, J., and Roy, C.R. (2009) *PLoS Pathog.*, **5**, e1000278.

121 Fernandez-Moreira, E., Helbig, J.H., and Swanson, M.S. (2006) *Infect. Immun.*, **74**, 3285–3295.

122 Nagai, H., Cambronne, E.D., Kagan, J.C., Amor, J.C., Kahn, R.A., and Roy, C.R. (2005) *Proc. Natl Acad. Sci. USA*, **102**, 826–831.

123 Kubori, T., Hyakutake, A., and Nagai, H. (2008) *Mol. Microbiol.*, **67**, 1307–1319.

124 Rowbotham, T.J. (1980) *J. Clin. Pathol.*, **33**, 1179–1183.

125 Chien, M., Morozova, I., Shi, S., Sheng, H., Chen, J., Gomez, S.M., Asamani, G., Hill, K., Nuara, J., Feder, M., Rineer, J., Greenberg, J.J., Steshenko, V., Park, S.H., Zhao, B., Teplitskaya, E., Edwards, J.R., Pampou, S., Georghiou, A., Chou, I.C., Iannuccilli, W., Ulz, M.E., Kim, D.H., Geringer-Sameth, A., Goldsberry, C., Morozov, P., Fischer, S.G., Segal, G., Qu, X., Rzhetsky, A., Zhang, P., Cayanis, E., De Jong, P.J., Ju, J., Kalachikov, S., Shuman, H.A., and Russo, J.J. (2004) *Science*, **305**, 1966–1968.

126 Steinert, M., Heuner, K., Buchrieser, C., Albert-Weissenberger, C., and Glöckner, G. (2007) *Int. J. Med. Microbiol.*, **297**, 577–587.

127 Isberg, R.R., O'Connor, T.J., and Heidtman, M. (2009) *Nat. Rev. Microbiol.*, **7**, 13–24.

128 Shin, S., and Roy, C.R. (2008) *Cell. Microbiol.*, **10**, 1209–1220.

129 Marcus, A.J., Broekman, M.J., Drosopoulos, J.H., Olson, K.E., Islam, N., Pinsky, D.J., and Levi, R. (2005) *Semin. Thromb. Hemost.*, **31**, 234–246.

130 Sansom, F.M., Newton, H.J., Crikis, S., Cianciotto, N.P., Cowan, P.J., d'Apice, A.J., and Hartland, E.L. (2007) *Cell. Microbiol.*, **9**, 1922–1935.

131 Sansom, F.M., Riedmaier, P., Newton, H.J., Dunstone, M.A., Müller, C.E., Stephan, H., Byres, E., Beddoe, T., Rossjohn, J., Cowan, P.J., d'Apice, A.J., Robson, S.C., and Hartland, E.L. (2008) *J. Biol. Chem.*, **283**, 12909–12918.

132 Banerji, S., Aurass, P., and Flieger, A. (2008) *Int. J. Med. Microbiol.*, **298**, 169–181.

133 Cirillo, S.L., Lum, J., and Cirillo, J.D. (2000) *Microbiology*, **146**, 1345–1359.

134 Liu, M., Conover, G.M., and Isberg, R.R. (2008) *Cell. Microbiol.* doi: 10.1111/j.1462-5822.2008.01180.x

135 Newton, H.J., Sansom, F.M., Bennett-Wood, V., and Hartland, E.L. (2006) *Infect. Immun.*, **74**, 1683–1691.

136 Newton, H.J., Sansom, F.M., Dao, J., McAlister, A.D., Sloan, J., Cianciotto, N.P., and Hartland, E.L. (2007) *Infect. Immun.*, **75**, 5575–5585.

137 Sedgwick, S.G., and Smerdon, S.J. (1999) *Trends Biochem. Sci.*, **24**, 311–316.

138 Mosavi, L.K., Minor, D.L., Jr., and Peng, Z.Y. (2002) *Proc. Natl Acad. Sci. USA*, **99**, 16029–16034.

139 Habyarimana, F., Al-Khodor, S., Kalia, A., Graham, J.E., Price, C.T., Garcia, M.T., and Abu Kwaik, Y. (2008) *Environ. Microbiol.*, **10**, 1460–1474.

140 Pan, X., Lührmann, A., Satoh, A., Laskowski-Arce, M.A., and Roy, C.R. (2008) *Science*, **320**, 1651–1654.

141 Molmeret, M., and Abu Kwaik, Y. (2002) *Trends Microbiol.*, **10**, 258–260.

142 Sutton, R.B., Fasshauer, D., Jahn, R., and Brunger, A.T. (1998) *Nature*, **395**, 347–353.

143 Sexton, J.A., and Vogel, J.P. (2004) *J. Bacteriol.*, **186**, 3814–3825.

144 Stone, B.J., and Kwaik, Y.A. (1999) *J. Bacteriol.*, **181**, 1395–1402.

145 Miyamoto, H., Yoshida, S., Taniguchi, H., and Shuman, H.A. (2003) *J. Bacteriol.*, **185**, 6712–6718.

146 Ochman, H., Lawrence, J.G., and Groisman, E.A. (2000) *Nature*, **405**, 299–304.

147 Doolittle, W.F. (1998) *Trends Genet.*, **14**, 307–311.

148 Eichinger, L., Pachebat, J.A., Glockner, G., Rajandream, M.A., Sucgang, R., Berriman, M., Song, J., Olsen, R., Szafranski, K., Xu, Q., Tunggal, B., Kummerfeld, S., Madera, M., Konfortov, B.A., Rivero, F., Bankier, A.T., Lehmann, R., Hamlin, N., Davies, R., Gaudet, P., Fey, P., Pilcher, K., Chen, G., Saunders, D., Sodergren, E., Davis, P., Kerhornou, A., Nie, X., Hall, N., Anjard, C., Hemphill, L., Bason, N., Farbrother, P., Desany, B., Just, E., Morio, T., Rost, R., Churcher, C., Cooper, J., Haydock, S., van Driessche, N., Cronin, A., Goodhead, I., Muzny, D., Mourier, T., Pain, A., Lu, M., Harper, D., Lindsay, R., Hauser, H., James, K., Quiles, M., Madan Babu, M., Saito, T., Buchrieser, C., Wardroper, A., Felder, M., Thangavelu, M., Johnson, D., Knights, A., Loulseged, H., Mungall, K., Oliver, K., Price, C., Quail, M.A., Urushihara, H., Hernandez, J., Rabbinowitsch, E., Steffen, D., Sanders, M., Ma, J., Kohara, Y., Sharp, S., Simmonds, M., Spiegler, S., Tivey, A., Sugano, S., White, B., Walker, D., Woodward, J., Winckler, T., Tanaka, Y., Shaulsky, G., Schleicher, M., Weinstock, G., Rosenthal, A., Cox, E.C., Chisholm, R.L., Gibbs, R., Loomis, W.F., Platzer, M., Kay, R.R., Williams, J., Dear, P.H., Noegel, A.A., Barrell, B., and Kuspa, A. (2005) *Nature*, **435**, 43–47.

149 Loftus, B., Anderson, I., Davies, R., Alsmark, U.C., Samuelson, J., Amedeo, P., Roncaglia, P., Berriman, M., Hirt, R.P., Mann, B.J., Nozaki, T., Suh, B., Pop, M., Duchene, M., Ackers, J., Tannich, E., Leippe, M., Hofer, M., Bruchhaus, I., Willhoeft, U., Bhattacharya, A., Chillingworth, T., Churcher, C., Hance, Z., Harris, B., Harris, D., Jagels, K., Moule, S., Mungall, K., Ormond, D., Squares, R., Whitehead, S., Quail, M.A., Rabbinowitsch, E., Norbertczak, H., Price, C., Wang, Z., Guillen, N., Gilchrist, C., Stroup, S.E., Bhattacharya, S., Lohia, A., Foster, P.G., Sicheritz-Ponten, T., Weber, C., Singh, U., Mukherjee, C., El-Sayed, N.M., Petri, W.A.J., Clark, C.G., Embley, T.M., Barrell, B., Fraser, C.M., and Hall, N. (2005) *Nature*, **433**, 865–868.

150 Gough, J. (2005) *Bioinformatics*, **21**, 1464–1471.

151 Brüggemann, H., Cazalet, C., and Buchrieser, C. (2006) *Curr. Opin. Microbiol.*, **9**, 86–94. Epub 2006 Jan 6.

152 Archer, K.A., and Roy, C.R. (2006) *Infect. Immun.*, **74**, 3325–3333.

153 Brieland, J.K., Jackson, C., Hurst, S., Loebenberg, D., Muchamuel, T., Debets, R., Kastelein, R., Churakova, T., Abrams, J., Hare, R., and O'Garra,

A. (2000) *Infect. Immun.*, **68**, 6567–6573.
154 Nagarajan, N.A., and Kronenberg, M. (2007) *J. Immunol.*, **178**, 2706–2713.
155 Tateda, K., Moore, T.A., Deng, J.C., Newstead, M.W., Zeng, X., Matsukawa, A., Swanson, M.S., Yamaguchi, K., and Standiford, T.J. (2001) *J. Immunol.*, **166**, 3355–3361.
156 Sporri, R., Joller, N., Albers, U., Hilbi, H., and Oxenius, A. (2006) *J. Immunol.*, **176**, 6162–6171.
157 Trinchieri, G. (2003) *Nat. Rev. Immunol.*, **3**, 133–146.
158 Brieland, J.K., Remick, D.G., LeGendre, M.L., Engleberg, N.C., and Fantone, J.C. (1998) *Infect. Immun.*, **66**, 65–69.
159 Archer, K.A., Alexopoulou, L., Flavell, R.A., and Roy, C.R. (2009) *Cell. Microbiol.*, **11**, 21–36.
160 Fortier, A., Diez, E., and Gros, P. (2005) *Trends Microbiol.*, **13**, 328–335.
161 Wright, E.K., Goodart, S.A., Growney, J.D., Hadinoto, V., Endrizzi, M.G., Long, E.M., Sadigh, K., Abney, A.L., Bernstein-Hanley, I., and Dietrich, W.F. (2003) *Curr. Biol.*, **13**, 27–36.
162 Molofsky, A.B., Byrne, B.G., Whitfield, N.N., Madigan, C.A., Fuse, E.T., Tateda, K., and Swanson, M.S. (2006) *J. Exp. Med.*, **17**, 1093–1104.
163 Ren, T., Zamboni, D.S., Roy, C.R., Dietrich, W.F., and Vance, R.E. (2006) *PLoS Pathog.*, **2**, e18.
164 Zamboni, D.S., Kobayashi, K.S., Kohlsdorf, T., Ogura, Y., Long, E.M., Vance, R.E., Kuida, K., Mariathasan, S., Dixit, V.M., Flavell, R.A., Dietrich, W.F., and Roy, C.R. (2006) *Nat. Immunol.*, **7**, 318–325.
165 Hägele, S., Kohler, R., Merkert, H., Schleicher, M., Hacker, J., and Steinert, M. (2000) *Cell. Microbiol.*, **2**, 135–171.
166 Solomon, J.M., Rupper, A., Cardelli, J.A., and Isberg, R.R. (2000) *Infect. Immun.*, **68**, 2939–2947.
167 Dorer, M.S., and Isberg, R.R. (2006) *Microbes Infect.*, **8**, 1637–1646.
168 Hilbi, H., Weber, S.S., Ragaz, C., Nyfeler, Y., and Urwyler, S. (2007) *Environ. Microbiol.*, **9**, 563–575.
169 Unal, C., and Steinert, M. (2006) *Methods Mol. Biol.*, **346**, 507–515.
170 Farbrother, P., Wagner, C., Na, J., Tunggal, B., Morio, T., Urushihara, H., Tanaka, Y., Schleicher, M., Steinert, M., and Eichinger, L. (2006) *Cell. Microbiol.*, **8**, 438–456.

7
The *Salmonella*–Mouse Interaction: A Versatile Model to Study Bacterial Infection

Jessica A. Thompson, Sophie Helaine, and David W. Holden

7.1
Introduction

Serovars of *Salmonella enterica* cause diseases in humans and other animals ranging from enteritis to more serious systemic infections that can be fatal if left untreated. *S. enterica* serovars Typhi, and Paratyphi A, B and C have a very narrow host range and cause typhoid fever only in humans and higher primates. Other serovars can infect a wider variety of hosts; for example, serovar Typhimurium causes enterocolitis in humans and a typhoid-like disease in some inbred, genetically susceptible mice such as the BALB/c strain. *Salmonella* infections are responsible for major disease and economic burdens worldwide: in 2004 it was estimated that there were 21.6 million cases with 216 510 deaths globally resulting from typhoid fever [1]. Complications in treatment have arisen due to the emergence of multi-drug resistance [2] and although two vaccines against typhoid are available, either purified Vi polysaccharide or a live attenuated strain, their efficacy is limited and revaccination is often required [3]. A greater understanding of the mechanisms underlying the disease could provide a basis for a rationally designed live-attenuated vaccine with improved efficacy, which might also be exploited to express heterologous antigens for cross-protection against other pathogens [4].

The host restriction of serovars causing human typhoid makes it difficult to establish the *in vivo* relevance of findings generated by *in vitro* studies. Hence, *S. typhimurium* infection of mice has been used extensively over several decades as a model for typhoid fever. This system is popular because it recapitulates several aspects of human typhoid, and both pathogen and host can be manipulated genetically with relative ease. Furthermore, the system has been exploited for the development of several useful techniques that have provided considerable insight into the molecular cell biology of bacterial virulence, the basis of host resistance and the physiology of the disease process. Some of these techniques are additionally important because of their applicability to other bacterial diseases.

Although infection of mice by *S. typhimurium* is a widely accepted model for typhoid, the similarities and differences between infection of humans by *S. typhi*

Bacterial Virulence: Basic Principles, Models and Global Approaches. Edited by Philippe Sansonetti
© 2010 WILEY-VCH Verlag GmbH & Co. KGaA, Weinheim
ISBN: 978-3-527-32326-5

and of mice by *S. typhimurium* should be borne in mind. The symptoms of typhoid fever in humans begin with a prolonged fever followed by inflammation of the lymphoid tissue of the small intestine. In one-third of cases, patients experience diarrhea. These symptoms occur after ingested *S. typhi* enter the small intestine and invade the Peyer's Patches. From there, they migrate into the mesenteric lymph nodes (MLNs) where growth occurs prior to release of bacteria into the bloodstream. Macrophages of the spleen, liver and bone marrow ingest the bacteria, which replicate in these organs, leading to hepatosplenomegaly. *Salmonella* eventually re-enter the blood following escape from the intracellular environment; as a consequence of the second bacteremia, infection spreads to the gall bladder, sometimes leading to reinvasion of Peyer's Patches in the distal ileum. This re-exposure causes inflammation and hemorrhagic necrosis of the Peyer's Patches, which can result in perforation of the small intestine, peritonitis, septicemia and death [5]. In approximately 1 to 6% of cases a chronic carrier state develops, where infection is asymptomatic despite evidence of colonization demonstrated by bacterial shedding in the feces.

Following oral administration of *S. typhimurium* to BALB/c mice, the bacteria move through the gastrointestinal tract with a small proportion able to colonize the mucosa of the distal ileum. Infected mice display an elevated temperature but do not develop diarrhea. Peyer's Patches are the main sites of invasion [6, 7] although adjacent enterocytes [8] and trans-cytosing CD18+ phagocytes [9] provide alternative means for breaching the epithelial barrier. The bacteria start to replicate and infection spreads to the MLNs draining these sites before progressing to the spleen and liver via the lymph and blood [10]. Ultimately, intra-macrophage growth within these organs results in enlargement of the spleen and liver, as occurs in cases of human typhoid fever. The resulting tissue damage has the potential to lead to organ failure, as well as a second, usually fatal, bacteremia. However, infection of the gall bladder and secondary exposure of the Peyer's Patches leading to perforation of the gut are not commonly associated with *S. typhimurium* infection of BALB/c mice.

In addition to the highly susceptible Nramp$^{-/-}$ BALB/c host (in which less than 10 intra-peritoneal-inoculated wild-type bacteria are sufficient to cause a lethal infection), genetically resistant *Nramp*$^{+/+}$ 129sv mice have been used to study a chronic disease characterized by persistence of *S. typhimurium* in macrophages within the MLNs for up to 1 year following oral inoculation [11].

The similarities in the infectious processes of *S. typhi* and *S. typhimurium* reflect extensive conservation of their genomes; several major virulence systems are common to the two serovars; these include the PhoP/Q regulon, and the *Salmonella* Pathogenicity Island (SPI)-1 and SPI-2 [12, 13]. Together these account for at least 100 virulence genes, although some variation exists among individual proteins of these systems. Approximately 4500 open reading frames (ORFs) exist within the genomes of Typhi and Typhimurium [14, 15], of which between 3 and 5% encode bona fide virulence factors [16, 17]. Analysis of the complete genome sequences has revealed that 13 and 11% of genes are unique to *S. typhi* and to *S. typhimurium*, respectively, when compared to that of the other serovar [14, 15]. The

S. typhi genome includes 200 pseudogenes; 145 of these are intact genes in *S. typhimurium*, some of which have been shown to be involved in virulence (e.g. *sseJ*). To some extent this may account for the differences between infections caused by the various serovars and their host range [14, 15]. Genetic differences between hosts are also likely to influence both host restriction and severity of the resulting disease.

These genetic differences (both between human and mouse, and *S. typhi* and *S. typhimurium*) introduce limitations to the use of the mouse model to study human typhoid disease. *S. typhi* genes potentially required for causing typhoid fever but not present in *S. typhimurium* (including several fimbrial operons of *S. typhi* that are not found in *S. typhimurium* [18])obviously cannot be studied in the mouse model. In the same way, some genes that contribute to the virulence of *S. typhimurium*, such as the *spv* operon, are absent from *S. typhi* and as such may not be relevant to the study of human typhoid [19]. As a result, findings from the mouse model must be extrapolated to infection of humans by *S. typhi* with caution and, if possible, corroboration.

Additional models of infection have been established to investigate other *Salmonella*-associated diseases. Bovines are natural hosts for *S. typhimurium*, displaying similar manifestations of enterocolitis as humans, but cattle are expensive, out-bred and much more difficult to manipulate than mice. As a result, a mouse model has been developed in which streptomycin pre-treatment followed by infection with *S. typhimurium* leads to enterocolitis [20, 21].

For the remainder of this chapter we consider the mouse model of acute typhoid. We describe the different ways in which it has been used, and how it has contributed to our understanding of the pathogenic process, from identification and characterization of virulence genes and host factors involved in infection, to detailed analyses of bacterial distribution and populations at the tissue and cellular levels.

7.2 Identification of Virulence Genes

7.2.1 *In vitro* Cell Models

To understand the disease process it is necessary to identify the molecules involved in pathogenesis and resistance, characterize their mechanisms of action and physiological effects, and integrate this knowledge with respect to the spatiotemporal progression of infection and the host response. There are several distinct environments during systemic infection in which different bacterial virulence factors are expressed and function to facilitate bacterial survival and growth. These include (i) the gut lumen, where bacteria must survive the low pH of the stomach and high osmolarity of the small intestine, before adhering to and invading the epithelium, (ii) the intracellular vacuole (or *Salmonella*-containing vacuole (SCV))

within epithelial cells and dendritic cells (DCs), (iii) the bloodstream and (iv) the SCV within macrophages, which are equipped to kill ingested bacteria by generating reactive oxygen and nitrogen, delivering antimicrobial peptides and the contents of lysosomes to phagosomes.

The availability of cell lines of epithelial, macrophage and DC origin, as well as primary macrophages and DCs from mice has led to innumerable *in vitro* studies on different facets of the infection process such as bacterial invasion, survival, replication, vacuole trafficking, immune modulation and cytotoxicity. They have provided major breakthroughs in our understanding of bacterial virulence: the identification of the SPI-1 type III secretion system and the *phoP-Q* regulon, for example [22–26]. Studies on *S. typhimurium* invasion and trafficking in epithelial cells have usually been carried out using human cell lines, such as HeLa, Henle-407 and HEp-2. One such screen with *S. typhimurium* and Henle-407 cells enabled *inv* genes encoding the SPI-1 type III secretion system to be identified [24]. Although this showed that these genes are necessary for bacterial invasion of human epithelial cells, the mouse model was then used to show decreased ability of *inv* mutants to colonize Peyer's Patches, and the small intestinal epithelium [24]. The *inv* mutants were attenuated when administered perorally to BALB/c mice, but not when given intraperitoneally, which showed that the locus does not have an important function during the systemic phase of infection [24].

Another important study used a large collection of *Salmonella* mutants to isolate strains with defects for survival and replication in elicited peritoneal macrophages from BALB/c mice. The screen of 9516 mutants yielded 115 with defects in intracellular survival, 83 of which were attenuated when subsequently tested in the mouse model of infection [22]. This provided strong indirect evidence that *Salmonella* must replicate in macrophages to cause disease, a concept that was not universally accepted around that time [27]. It also generated many interesting mutants for further analysis, including one carrying a mutation in *phoP*, a key regulator of expression of *Salmonella* virulence genes [23, 26].

Although much valuable information can be gained from the use of *in vitro* screens for bacterial virulence genes, they have an obvious limitation: they usually focus on one specific aspect of the disease process, ignoring the broader context of these individual interactions in pathogenesis. Bacteria respond to different cell types and changing conditions in the host as infection progresses. This occurs in the face of a developing immune response, which involves different cell types whose actions are subject to complex temporal and spatial regulation. Unfortunately it is currently not possible to simulate these conditions *in vitro*.

7.2.2 *In vivo* Screens for Virulence Genes

To circumvent the limitations of screening *in vitro* for *Salmonella* virulence factors, several techniques have been developed to identify genes that are either expressed or required for growth within the mouse. Alongside the obvious benefits of increased physiological relevance due to studying the entire bacteria–host interac-

tion, some of these methods enable pools of bacterial strains to be tested in the same mouse. The number of animals required for analysis is reduced, with clear benefits: ethically, and due to the resulting reduction in host-to-host variation.

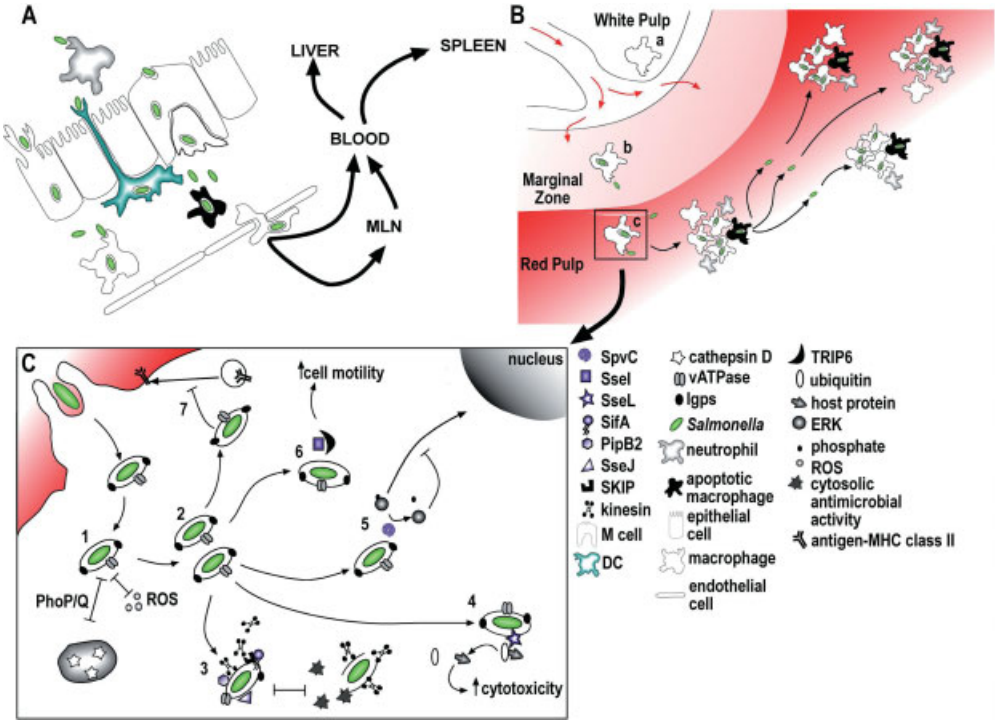
The first use of complex pools of *Salmonella* strains in the mouse was a groundbreaking study by the Mekalanos group [28]. They developed *in vivo* expression technology (IVET), in which genes expressed selectively in infection are identified using a genome-wide promoter trap screen. In the original version, partially digested genomic DNA from *Salmonella* was ligated into a plasmid upstream of a promoterless *purA* gene required for purine biosynthesis and bacterial growth within the host. A library of *Salmonella purA* mutants each carrying a different plasmid was injected into mice and complementation of auxotrophy positively selected for virulent strains. These were recovered and tested *in vitro* to ensure that the promoters were only expressed in the host, and genes were identified following plasmid sequencing. This work generated widespread interest, and was a crucial development in the study of bacteria–host interactions. Arguably, this was not so much because of the genes identified, but because it stimulated the development of several other screening approaches, both in *Salmonella* and other pathogens, for *in vivo* induced genes [29] and also for high throughput analysis of conventional mutants [30].

One shortcoming of the original IVET method is that transient or low level expression of *ivi* genes can be missed. This was addressed by the development of recombinase-based IVET (RIVET) [29]. In this case, the promoterless *pur* gene on the IVET vector is replaced with a resolvase gene (*tnpR*), with *lacZY* encoded downstream. A tetracycline resistance cassette flanked by *res1* sites is introduced into the bacterial chromosome. Following generation of reporter fusions and their expression upon infection of the host, expression of the resolvase catalyzes recombination events at the *res1* sites; the tetracycline resistance cassette is removed and the bacterial strain changes from a tet^R to tet^S phenotype. This phenotypic switch is detected by replica plating; use of selective media in conjunction with *lacZY* also enables elimination of *in vitro* expressed fusions. RIVET was originally developed using *Vibrio cholerae*, but a 'directed' application of RIVET using *Salmonella*-infected mice has been used to assess the expression of an Ni/Fe uptake-type hydrogenase in different organs [31], and to show that expression of SPI-2 can be detected during initial stages of infection in the lumen of the intestine [32].

A fluorescence-based method (differential fluorescence induction) has also been developed for the isolation of bacterial genes expressed during infection of host cells. Macrophages infected with *Salmonella* carrying a plasmid with chromosomal fragments fused to a GFP gene reporter were separated by fluorescence-activated cell sorting (FACS) and bacteria were then isolated by FACS on the basis of reduced fluorescence *in vitro* [33]. As well as showing that SPI-2 gene expression is strongly induced following uptake by host cells [34] this technique also provided a construct for constitutive expression of GFP by *Salmonella* (pFPV25.1) which has proved very useful for detecting intracellular *Salmonella in vitro* and *in vivo* [33, 35].

IVET and its variants have revealed genes which are expressed in the host, but it is important to note that expression does not always correlate with a requirement for growth *in vivo*, or with a role in virulence: if the *in vitro* expression analysis involved growth in rich media, the nutritionally limited environment *in vivo* might identify biosynthetic genes involved in general metabolism rather than virulence genes.

Signature-tagged mutagenesis (STM) was developed as a means for high throughput identification of *Salmonella* virulence genes, using the mouse to select against transposon mutants with *in vivo* growth defects [30]. Each transposon carries a different DNA barcode (signature tag) flanked by identical DNA sequences. This enables the tags from a pool of mutants to be co-amplified, labeled and hybridized to DNA from individual mutants arrayed on filters. Hybridization patterns were generated by tags amplified from pools of 96 mutants before inoculation and following bacterial recovery from infected organs. The signature tags therefore act as molecular barcodes to detect the presence of each mutant in the pools. STM has its own limitations, but many improvements to the technique have been made using both bacteria and fungi and these have recently been discussed elsewhere [36].



The initial application of STM resulted in the discovery of the *Salmonella* Pathogenicity Island (SPI)-2 encoded Type Three Secretion System (T3SS) [30, 37]. This T3SS delivers over 20 effector proteins across the vacuolar membrane of infected cells. These have many different functions, including the promotion of bacterial replication [38, 39], avoidance of killing by reactive oxygen [40] and nitrogen species [41], modulation of immune functions in macrophages and DCs [42–44], control of vacuolar membrane dynamics [45–47], induction of host cell cytotoxicity [48–50] and stimulation of macrophage motility [51], as summarized in Figure 7.1C. Together these make a major contribution to systemic growth of bacteria in the mouse [30, 39]. The SPI-2 TTSS of *S. typhi* is also important for intracellular growth [4] and a strain incorporating an SPI-2 mutation appears to be safe and a potential single oral dose vaccine for human typhoid fever [52].

Figure 7.1 Interactions between *Salmonella* and the murine host. (A) The intestinal phase of infection. Fimbriae, flagella and alterations to LPS, among other bacterial factors, promote survival and attachment of *Salmonell*a in the gut lumen and to the intestinal wall in the face of host responses including antimicrobial peptides, phagocytes, complement and peristalsis. *Salmonella* traverses the intestinal barrier by three mechanisms: (i) SPI-1 T3SS-mediated invasion of M cells, (ii) SPI-1 T3SS-mediated invasion of adjacent enterocytes, and (iii) phagocytosis and translocation by CD18+ phagocytes. In the sub-epithelial dome, *Salmonella* are phagocytosed by macrophages and DCs. SipA can induce SPI-1 T3SS-associated caspase-1-mediated host cell death, alternatively, cells undergo maturation and migration processes, either traveling via the lymph to the MLNs, or directly into the blood, before passing to the liver and spleen. (B) Interactions in the spleen. *Salmonella* are predominantly found in marginal zone (b) and red pulp (c) macrophages, with very little colonization of white pulp metallophilic marginal zone (a) macrophages. Bacterial replication is predominantly intracellular, granuloma formation occurs with recruitment of further phagocytes. Net growth of *Salmonella* in the liver and spleen appears to occur through death of infected cells, release of intracellular bacteria and the establishment of new foci of infection, not the expansion of those already in existence. (C) Intra-macrophage interactions. (1) *Salmonella* is phagocytosed by macrophages, and resides within a membrane-bound vacuole: the *Salmonella*-containing vacuole (SCV). The SCV acidifies following acquisition of v-ATPase, acquires certain late endosomal markers but avoids fusion with lysosomes and delivery of ROS generated by the oxidative burst. (2) The SPI-2 T3SS is activated, effectors are delivered into the macrophage cytosol and bacterial replication is stimulated. (3) Translocated SifA and PipB2, in conjunction with the host protein SKIP, regulate the levels of kinesin on the SCV. This, alongside the activity of the effector SseJ, maintains membrane integrity, preventing exposure to and killing by cytosolic antimicrobial activity. (4) SseL deubiquitinates host proteins and stimulates host cell death, whilst the phosphothreonine lyase activity of SpvC (5) inactivates MAP kinases such as ERK, reducing translocation of activated ERK to the nucleus. (6) SseI binds the host protein TRIP6 and increases motility of the host cell. As yet unknown SPI-2 T3SS-mediated activity inhibits antigen presentation on MHC class II (7).

7.3 Analyzing Gene Function *in vivo*

7.3.1 Measuring the Contribution of Bacterial Genes to Virulence

The majority of the 150–200 predicted virulence genes in the *S. typhimurium* genome have now been identified. However the specific biochemical functions of many of these remain to be determined. If the predicted amino acid sequence of a gene does not provide a clue to its function, phenotypic analysis of the mutant strain can be used to gain insights into the physiological role of the gene in question. A useful starting point is to determine if loss of the gene results in attenuation of virulence. Historically, the level of virulence attenuation conferred by the mutation was investigated by inoculation of groups of mice with a range of doses of inocula of a single strain. The LD_{50} (or median lethal dose), defined as the bacterial dose that causes 50% of the animals to reach the experimental end-point (a defined stage of morbidity or death) was recorded and compared between wild-type and mutant strains. This is a relatively crude method in that it reflects only the cumulative effect of all steps involved in causing lethality. Furthermore, it is expensive due to requirements for a large number of animals and raises ethical considerations if the chosen endpoint causes undue suffering to the host. Instead of using death or indicators of morbidity to define an experimental end-point, it is of course possible to sacrifice mice at various time points after inoculation and determine the bacterial load from different organs. Bacteria can easily be recovered from the Peyer's Patches, MLNs, spleen and liver, and the numbers of viable bacteria enumerated by colony forming units (CFU) following plating on laboratory medium. Differences in the numbers of mutant and wild-type bacteria from these sites provide a measure of attenuation of the mutant strain and can also indicate whether the gene has a tissue- or organ-specific function. In addition, administering bacteria orally, intraperitoneally or intravenously can generate further information about the spatial requirements for the virulence factor within the host. As indicated above, *Salmonella* strains mutated in the SPI-1 T3SS, which is required for colonization and translocation of the intestinal epithelium, are attenuated when administered orally, but not following intraperitoneal inoculation, showing that this secretion system is dispensable once the barrier of the intestinal epithelium has been surmounted [24]. On the other hand, strains carrying mutations in SPI-2, *phoP* and *spv* genes are attenuated following both oral and intraperitoneal injection [53, 54], which shows that they are required for systemic infection, but does not reveal whether they are required for colonization of the gut epithelium, Peyer's Patches or MLNs. Modification of inoculum size and duration of infection can also shed light on virulence gene function.

A limitation of single strain infections is that the use of multiple animals introduces host-to-host variation and this in turn necessitates large numbers of animals to establish the significance of any results obtained. As a result, the competitive index (CI) test has largely supplanted single strain infections as a means

of measuring virulence attenuation. In the CI test, equal numbers of the wild-type and mutant strains are combined in the inoculum and a mixed pool of bacteria is subsequently recovered from organs of infected mice; individual strains can be distinguished on the basis of different antibiotic resistance markers. The CI is defined as the ratio between the mutant strain and the wild-type in the output divided by the ratio of the two strains in the input; ratios of less than 1 indicate a competitive disadvantage of mutant relative to wild-type; a CI equal to 1 demonstrates no effect of the mutation upon virulence; a ratio of more than 1 indicates an advantage for mutant over wild-type [55, 56].

The CI is a much more sensitive measure of virulence attenuation than the LD_{50} because it provides a direct readout of overall bacterial growth compared to an indirect effect on mouse morbidity or survival. Furthermore, the elimination of host-to-host variation reduces the number of mice required to achieve statistical significance. A theoretical drawback exists in that the defect of some attenuated strains might be complemented in *trans* by the presence of wild-type bacteria in the same tissue. This does not seem to be a major problem in practice as the majority of *Salmonella* mutants that are attenuated when tested singly are also attenuated in mixed infections. The CI is a versatile method, where route of inoculation, dose, strain combination and ratio, time and site of recovery can all be varied to provide a detailed description of the spatiotemporal contribution to virulence of a given gene. All these factors have made CI analysis a very popular approach for investigating *Salmonella* virulence.

To cause infection, *Salmonella* must (i) survive the killing mechanisms of the host, and then (ii) acquire nutrients and membrane in order to replicate in SCVs within macrophages. Although the distinction between these two activities is not always clear, some virulence gene functions can be broadly classified as 'enabling avoidance of killing' while others are involved in 'facilitating replication'. For example, the PhoP/Q regulon is required for avoidance of killing, since its induction increases bacterial resistance to antimicrobial peptides [23], whereas some genes involved in metabolite acquisition facilitate bacterial replication [57, 58]. Gulig and Doyle [59] used segregation of a temperature-sensitive (ts) plasmid to establish whether the *Salmonella* virulence plasmid contributes to replication or avoidance of killing *in vivo*. The ts plasmid fails to replicate at 37 °C and therefore becomes diluted in the bacterial population as it divides in host tissues. If an attenuating mutation causes reduced growth rate then a greater proportion of the mutant population recovered from infected mice will carry the ts plasmid. Studies with both *spv* [59] and SPI-2 mutants [39] strongly suggest that these two virulence systems function to increase replication rather than help the pathogen to avoid killing by the host.

7.3.2
Investigating Interactions between Bacterial Virulence Genes

The CI test has been adapted for analysis of virulence gene interactions. Baumler *et al*. were the first to use mixed infections of *Salmonella* strains to gain evidence

for functional independence or synergism between individual genes [60]. The CIs of wild-type versus Δ*ipfC* (fimbrial gene), wild-type versus Δ*invA* (SPI-1 T3SS apparatus) and wild-type versus Δ*ipfC*Δ*invA* were compared and showed evidence of phenotypic additivity of Δ*ipfC*Δ*invA* mutations, suggesting an independence of function for *ipfC* and *invA*. This work provided the basis for a modified CI test, referred to as canceled-out index (COI) [61, 62]. In brief, CI values are obtained for single mutants in comparison to wild-type and the ratios are then obtained for single mutants relative to the double mutant. If two virulence genes act independently, then it is assumed that the contributions to virulence (and therefore the attenuation upon removal of these factors) will be additive, with a COI for Δ*geneA* versus Δ*geneA*Δ*geneB* similar to that for wild-type versus Δ*geneB*. In contrast, if the two genes have a linked function, any effects on virulence that would normally be caused by Δ*geneB* when in isolation are abrogated by the presence of Δ*geneA* in both strains. No selective advantage exists for the single mutant strain over the double mutant; in this case the COI would be expected to be close to 1.0. The principles of COI were illustrated by demonstrating a lack of interaction between *purD* (involved in purine biosynthesis) and *ssaV* (encoding a structural component of the SPI-2 TTSS) but functional linkage for the latter and *sseB* (encoding part of the T3SS translocon required for effector delivery into the host) [62].

7.3.3
Investigating Interactions between Host and Pathogen Factors

The availability of mutant mouse strains affected in immune functions has vastly increased over recent years. These mutant strains, generated either by conventional breeding, or targeted or random mutagenesis, can be utilized in the same way as wild-type mice to compare LD_{50}, bacterial load in various organs, CI and COI, for both wild-type and mutant bacteria. Examples of the numerous studies carried out and their main findings are summarized in Table 7.1.

Infection of wild-type and mutant mice with wild-type *Salmonella* and comparison of either LD_{50} or bacterial load at different time points enables bacterial virulence to be determined in the presence or absence of specific host factors. If increased morbidity or bacterial growth is observed in the mutant mouse compared to wild-type, then the gene in question is presumed to have a role in resistance to *Salmonella*, by limiting replication or through active killing of the pathogen. Alternatively, loss of a host factor can inhibit *Salmonella* growth, suggesting either a direct role in promoting virulence (e.g. the host factor might be an interacting partner for a bacterial virulence protein), or non-specifically through an involvement in immune function that is exploited by *Salmonella* for growth.

Manipulation of the mouse model is not restricted to knock-outs, transgenics or conventional breeding: it has also been possible to remove certain cell types and study their involvement in the disease process. Approaches that have been used include irradiation and antibody treatment. A protective role for neutrophils was demonstrated by the use of mice rendered neutropenic by antibody-mediated depletion; these animals exhibited increased bacterial colonization of systemic

Table 7.1 Examples of the use of mouse gene knockouts to investigate host responses to *Salmonella* infection.

Gene knock-out	Gene function	Major finding	Reference
gp91phox$^{-/-}$	Generation of ROS	Contributes to early macrophage killing of Salmonella; knock-out mice exhibit increased bacterial numbers by day 1 in spleen and liver following i.v. inoculation and succumb more rapidly to infection	[40, 63, 64]
iNOS$^{-/-}$	Generation of RNS	RNS contribute to early and late macrophage anti-Salmonella activity, mediating a late stage bacteriostatic effect in the spleen and liver; granulomas form but are unable to contain bacterial replication	[40, 63]
phox$^{-/-}$iNOS$^{-/-}$		ROS and RNS make distinct, additive contributions to bacterial killing in peritoneal macrophages	[63]
Ity (nramp1$^{-/-}$)	Divalent cation transporter on phagosome membrane	nramp1 null mice behave as Ity^{s} mice, succumbing to infection 4–6 days after i.v. inoculation, attributing the resistance associated with this locus to this gene. Mutation in conjunction with NOS2A (RNS) or Cybb (ROS) demonstrates additive (independent) effects upon bacterial growth and resistance to infection	[65, 66]
FcγRI$^{-/-}$ FcγRII$^{-/-}$ FcγRIII$^{-/-}$	IgG receptors	Survival of knock-out mice is reduced upon secondary infections with orally administered virulent Salmonella	[67]
Igh-6$^{-/-}$	Ig heavy chain: null mice lack peripheral B cells	Lack of peripheral B cells has no effect on host survival in primary infections with virulent Salmonella, or bacterial growth in spleen and liver of attenutated strains. Decreased host survival occurs upon secondary exposure to virulent Salmonella following initial infection with attenuated strain, thought to be due to impaired Th1 responses	[63, 68]

Table 7.1 *Continued*

Gene knock-out	Gene function	Major finding	Reference
Igμ$^{-/-}$	Lack B cells	Increased oral LD_{50} in absence of mature B cells and antibodies	[69]
C1qa$^{-/-}$	Early component in classical complement pathway	Increased susceptibility to Salmonella infection, with increased bacterial growth in the spleen and liver. *In vitro* infected c1qa$^{-/-}$ macrophages contain more Salmonella per cell	[70]
caspase-1$^{-/-}$	Cysteine protease, involved in Salmonella-induced host cell death	Increased susceptibility to infection, with increased bacterial recovery from Peyers Patches, MLN, spleen and liver. Caspase-1 cleaves and maturates IL-1β and IL-18. Exogenous IL-18 partially restores bacterial growth in caspase-1$^{-/-}$ mice to levels in wild-type	[71]
tlr4$^{-/-}$lbp$^{-/-}$cd14$^{-/-}$	Receptor and associated molecules: bind LPS	Delayed cytokine production, decreased NO and cellular responses including macrophage-mediated killing. Increased bacterial growth in Kupffer cells. Recapitulation of the nramp$^{-/-}$ phenotype. TLR4-mediated signaling important for initiation of innate immune responses	[72–75]
tlr5$^{-/-}$	Receptor: binds flagellin	TLR5 on laminar propria CD11c+ phagocytes aids Salmonella translocation from the intestinal lumen to MLN. Mice succumb less rapidly to oral infection with lower bacterial counts in the MLN, spleen and liver	[76]
tlr2$^{-/-}$	Receptor: binds peptidoglycan	In conjunction with tlr4$^{-/-}$: increased susceptibility to infection with increased bacterial growth in bone marrow-derived macrophages and spleen; decreased host survival (tlr2$^{-/-}$ alone does not affect Salmonella virulence in the host)	[75]
myd88$^{-/-}$	Signaling adapter protein for TLRs	Similar phenotype to tlr2$^{-/-}$tlr4$^{-/-}$ knock-out mice: signaling via MyD88 is important for resistance to Salmonella	[75]

Table 7.1 *Continued*

Gene knock-out	Gene function	Major finding	Reference
irgm1$^{-/-}$	LRG-47 (p47 GTPase), IFN-inducible, immune function	Increased susceptibility to infection with increased bacterial load in spleen and liver, defects in granuloma maturation and decreased macrophage accumulation in spleen. IFN-γ-treated irgm1$^{-/-}$ macrophages are less able to restrict bacterial growth	[77]
cd154$^{-/-}$	T cell surface molecule: CD40 interaction	Hypersusceptibility to attenuated Salmonella	[78]
T-bet$^{-/-}$	Transcription factor: Th1 cell differentiation	Decreased host survival upon infection with attenuated Salmonella; increased splenic bacterial burden, disrupted cytokine responses	[79]
ubp43$^{-/-}$	Negative regulator of type I IFN responses	Fewer bacteria recovered from spleen and liver of mice; only slight increase in host survival due to hypersensitivity to LPS	[80]
cav-1$^{-/-}$	Caveolin-1	Increased susceptibility to Salmonella infection, increased bacterial burden in spleen and liver. Increased production of inflammatory cytokines, chemokines and NO, probably from macrophages thought to be involved in heightened sensitivity of KO mouse	[81]
tnfα$^{-/-}$tnfαp55r$^{-/-}$	TNFα and receptor	tnfα$^{-/-}$ peritoneal macrophages: increased growth of Salmonella with decreased NO responses. Receptor KO mice: succumb to oral infection more rapidly, greater splenic and hepatic burdens, impaired immunity to secondary infections following inoculation with an attenuated strain	[82]
il-4$^{-/-}$	IL-4: induction of Th2 response	Knock-out mice succumb more slowly to infection	[83]

sites [84]. The contributions of certain cytokines have also been investigated using antibodies that bind either the receptor in a non-activatory manner or the cytokine itself, thereby abrogating the activity of these molecules. For example, neutralization of IL-12 [85] or IL-18 [86, 87] increased the growth of *Salmonella* in the mouse reticuloendothelial system, and this was accompanied by decreased IFN-γ production and increased host morbidity. Neutralizing antibodies can be very useful when the corresponding gene knock-out causes lethality or developmental defects, and overall these studies have been extremely important in developing our understanding of the basis of resistance to *Salmonella* in the mouse.

Further understanding of the contributions of host resistance mechanisms to *Salmonella* infection has come from the use of *gp91phox*$^{-/-}$ and *iNOS*$^{-/-}$ mice, which are unable to generate ROS or RNS, respectively [63]. Although both mutant mouse strains had increased susceptibility to infection, ROS were shown to have an important bactericidal effect during the early, acute phase of infection, whereas the contributions of RNS appeared to be more bacteriostatic, mediating protection later on.

It has been known for many years that the *Ity* locus (mutated in BALB/c mice) mediates protection against early growth of *Salmonella*, but how the encoded protein (Nramp1/Slc11a1, a divalent cation transporter localized on the phagosomal membrane) actually contributes to elimination of the pathogen at a cellular or molecular level remains unclear. Introduction of wild-type *Nramp1* cDNA into RAW264.7 macrophages completely blocked intracellular replication of *S. typhimurium* [88]. Nramp1/Slc11a1$^{-/-}$ knock-out mice have also been used to show that the Slc11a1 protein contributes to the production of pro-inflammatory cytokines by dendritic cells [89], and to the development of inflammation and protection against *S. typhimurium* in the enterocolitis model in mice [90].

Combining *Salmonella* mutants with mouse mutants provides another means for revealing aspects of the infectious process. The role of CD18+ phagocytes in extraintestinal dissemination of *Salmonella* was unveiled in this way: the SPI-1 T3SS is required for bacterial invasion of intestinal epithelial cells, but the attenuation of virulence of SPI-1 mutants following oral inoculation is not complete [24]–this suggested alternative routes of entry across the gut epithelium. The amount of systemic colonization by SPI-1 null mutant bacteria was further reduced in CD18 knock-out mice following oral but not intra-peritoneal inoculation [9]. This provided very strong evidence of a SPI-1 independent, CD18-dependent route of transmission from the intestine of infected animals, possibly via CD18-expressing phagocytes translocating from the gut into the bloodstream and on to the spleen and liver [9] (Figure 7.1A). Later work using immunohistochemical and two-photon imaging of explanted gut tissue containing GFP-expressing CD11c cells from infected mice revealed trans-epithelial DC extensions along the length of the ileum [91]. MyD88 and TLR-dependent signaling stimulates dendritic cells to extend dendrites through the epithelial layer; the formation of tight-junction-like interactions between dendritic cells and epithelial cells prevents disruption of barrier integrity. These protrusions are retractable, increased upon infection with *Salmonella*, and were shown to contain the pathogen. This supports the hypothesis that DCs sample the gut lumen at sites distant from Peyer's Patches [91] and that they provide an important SPI-1-independent means of access to systemic sites [92–94].

Mutant mice can also be used to establish functional links between pathogen and host gene products, by comparison of the growth and effects of wild-type and mutant *Salmonella* in wild-type and mutant mice. For example, the increased virulence of SPI-2 T3SS mutant bacteria in *phox*$^{-/-}$ knock-out mice compared to wild-type animals provided the first indication that one function of this secretion system is to restrict delivery of ROS to SCVs in macrophages [40]. This approach is not limited to the study of individual mutants: an application of STM, known as differential STM, was initially developed for *Mycobacterium* [95] and then applied to *Salmonella*. The technique involves inoculation of the same bacterial mutant library into wild-type and mutant mice in search of mutant strains whose attenuation in the wild-type mouse is rescued by the lack of a particular factor in the mutant host. In the *Salmonella* study, *iNOS* and *phox*$^{-/-}$ knock-out mice were used. In the *phox*$^{-/-}$ background an attenuated strain was rescued that was mutated in a gene (*ydiV*/*cdgR*) encoding an EAL domain-containing protein predicted to be a cyclic diGMP phosphodiesterase [96]. Subsequent analysis indicated that the presence of *ydiV*/*cdgR* lowered intrabacterial levels of cyclic diGMP, an important regulator of diverse physiological processes, including bacterial virulence gene expression. Reduced levels of cyclic diGMP were associated with reduced killing of *Salmonella* by macrophages and promoted *in vitro* resistance of bacteria to hydrogen peroxide [96], thereby explaining the recovery of the mutant strain in mice lacking the ability to generate an oxidative burst.

Although these approaches can indicate a functional link between host and pathogen factors, initial results must be interpreted with caution. If the bacterial mutant recovers the ability to behave as wild-type in the mutant host, that provides prima facie evidence for a functional link between both elements. However, loss of certain host factors might promote non-specific replication and increased survival and/or replication of both mutant and wild-type bacteria. If rescue of mutant bacteria is measured by a hybridization signal, or mouse morbidity or mortality, the increased load or altered pathology, of both strains in the mutant mouse, might not be detectable. In this scenario there would not necessarily be a functional link between the bacterial gene and host gene, but a more general role for the host factor in resistance to *Salmonella*. Therefore it is prudent to carry out follow-up studies *in vivo* by examining bacterial loads in different tissues and associated pathology, to ensure that specific rescue of the mutant strain occurs as a result of loss of the corresponding host factor.

7.4
In-depth Analysis of Cell Interactions within Host Tissues

7.4.1
Distribution of Salmonella

The widespread interest in the *Salmonella*–mouse model of infection in terms of pathogenesis, immunology and vaccinology, has provided a very detailed

description of the route of infection, the tissue and cellular distribution of bacteria, their population dynamics and the sites of action of various virulence factors.

A seminal study by Carter and Collins established the primary site of *Salmonella* invasion: sections of the gastrointestinal tract were injected with a dye to indicate regions drained by lymph nodes. Following infection, the draining lymph nodes were recovered to determine those that contained viable bacteria and therefore the section of the gut from which they originated. Although the cecum and large intestine were exposed to large numbers of bacteria, viable *Salmonella* were only found in the MLNs that drained the distal ileum [10]. They concluded that *Salmonella* were able to invade the mucosa and Peyer's Patches of the distal ileum, with colonization proceeding through the lymph to the draining lymph nodes and then to the liver and spleen.

A fuller understanding of the main sites of invasion of the intestinal epithelium by *Salmonella* was provided by studies involving microscopy of sections taken from infected murine ileal ligated loops. Confocal, scanning electron and transmission electron microscopy revealed interactions between *S. typhimurium* and M cells found in the follicle-associated epithelium overlying Peyer's Patches [6–8]. M cells actively ingest antigens, bacteria and viruses from the gut lumen; their deeply invaginated basolateral surface allows close proximity of lymphocytes and dendritic cells to the apical surface, enabling efficient transport of ingested material to the underlying lymphoid tissue. While the phagocytic ability of M cells accounts for some degree of *Salmonella* uptake [7, 97], it can also occur via a bacterially-driven process: invasive SPI-1 T3SS-expressing *Salmonella* appear to be able to target, invade and then destroy M cells [6, 8], thereby gaining access to the sub-epithelial layer. Neighboring enterocytes were also invaded to a lesser extent in an SPI-1 T3SS-dependent manner [8] (Figure 7.1A).

Light-emitting and fluorescent bacteria have also been tracked by microscopy and FACS to provide information about their distribution in both intestinal and systemic phases of infection. Contag *et al*. used *Salmonella* expressing luciferase to follow bacterial distribution in living hosts [98]. Different outcomes of infection dependent upon both host and pathogen genotype were demonstrated: resolution, persistence and progression. However, no bioluminescence could be detected from the spleen or liver despite these organs being major sites of bacterial replication. Photonic detection of bacteria in this system was limited by several factors including the opacity and light absorbance properties of different mouse tissues, the relatively high number of bacteria required for detection of bioluminescence, and the necessity for aerobic environments for this light emission to occur.

Antibody labeling and confocal microscopy have been used to show the presence of *Salmonella* in macrophages in the liver, and their cytotoxic effects [99]. GFP-expressing *Salmonella* are now used routinely to detect bacteria in infected tissues and cells. Hopkins *et al*. infected murine ileal ligated loops with attenuated GFP-expressing *Salmonella* and used antibody staining and confocal microscopy to demonstrate that bacteria resided within CD11c+ dendritic cells in Peyer's Patches [100]. To establish the cell types occupied by *Salmonella* during growth in the spleen, Salcedo *et al*. infected mice with bacteria expressing GFP from pFPV25.1, which

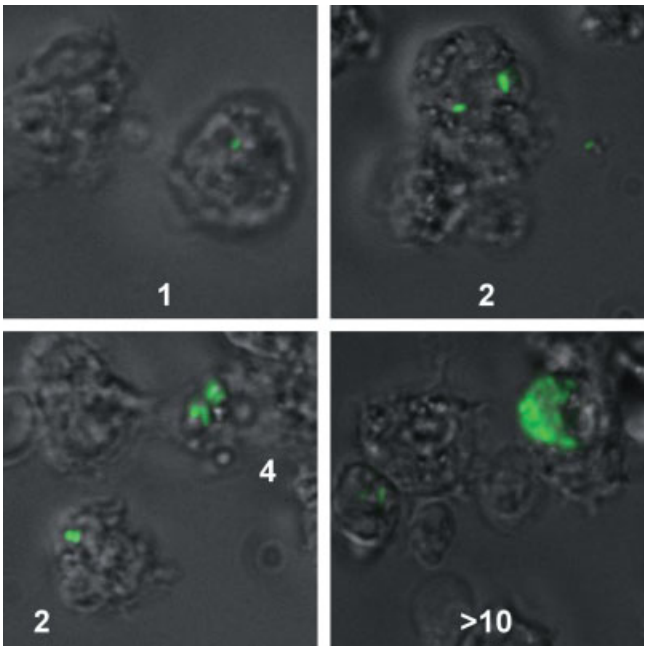


Figure 7.2 *In vivo* heterogeneity of intracellular numbers of bacteria in splenocytes from BALB/c mice. Characteristic images of splenic CD11b+ macrophages extracted from mice infected with *S. typhimurium* expressing GFP from pFPV25.1 are shown. Heterogeneity of infection is clearly observable, with macrophages containing 1, 2, 4 and >10 bacteria.

is stable and does not have a significant effect on virulence [33, 35]. Intra-macrophage bacteria were observed by confocal microscopy of splenic cell suspensions. FACS was used to quantify the distribution of bacteria within macrophage subsets: both red pulp and marginal zone macrophages were colonized and a disproportionately high number were found in Scavenger Receptor/2F8-positive macrophages; very few were located in the relatively non-phagocytic marginal metallophilic macrophages [35] (Figure 7.1B). Reduced replication of SPI-2 null mutant bacteria in these macrophages was observed, and it was also possible to show that the loss of vacuolar membrane around a Δ*sifA* mutant strain [101] also occurs *in vivo*. At late stages of infection, the numbers of bacterial cells per macrophage were very heterogeneous, with roughly 20% of infected cells harboring one bacterium and a similar percentage harboring over 10 bacteria (Figure 7.2). Developing a method of two-color FACS analysis of *in vivo*-infected cells to better distinguish between bacterial GFP fluorescence and the high levels of autofluorescence generated by host tissues, Bumann *et al.* first analyzed bacterial gene expression within the host through promoter fusions with GFP. This study detected SPI-1 (P*sicA*) expression in the intestinal lumen and Peyer's Patches in the first 24 h of infection, with a later and more long-lasting activity for P*ssaH* (SPI-2) [102]. This method was then used to sort for differentially infected cells from the spleen, confirming both the

existence of heterogeneity in intracellular numbers of *Salmonella*, and the predominance of marginal zone and red pulp macrophages in the infected pool [103].

In vivo delivery of SPI-2 effectors into host cells was detected using fusion proteins that generated a blue signal following translocation. FACS analysis showed the presence of four effectors in the cytoplasm of splenocytes [104]. Surprisingly, this study also reported that neutrophils are a major site of intracellular *Salmonella*; in view of the other work on this subject [35, 103], further research may be required to resolve the issue of whether macrophages or neutrophils are the predominant intracellular niche for splenic *Salmonella*.

7.4.2 Population Dynamics of Bacteria in the Murine Host

Infection of BALB/c mice by *Salmonella* is both highly dynamic and complex, in that both replication and killing of bacteria occurs during the process of bacterial spread between organs and within tissues and cells of the same organ. Increased understanding of these processes will help to identify critical points in the infection and resistance processes, potentially shedding light on why some infections lead to aggressive, systemic disease, some to clearance of the pathogen, and others to chronic, asymptomatic infection. One of the first studies of bacterial population dynamics in murine typhoid was carried out by Meynell and Stocker. Despite inoculating mice with equal numbers of two *S. typhimurium* variants, they were only able to find one of these present by the time of terminal bacteremia [107], thus concluding that escape into the bloodstream and replication resulted from a chance event and was possible from just a single bacterial cell. This appeared to be stochastic; they concluded that it was dependent on different microenvironments to which individual bacteria were exposed during infection. At high dose inocula, the ratio between the strains in the blood after infection was the same as their ratio in the inoculum. This implies that if the inoculum dose is sufficiently high, systemic infection of mice results from the multiplication of many of the cells present in the inoculum, rather than the clonal expansion of a small number, which in turn could be due to a breakdown in host resistance.

In a more recent study by Sheppard *et al.*, two strains (distinguished by differing LPS O-antigens) were used to show that foci of infection in the liver developed from the clonal expansion of a single bacterium without mixing between foci. Microscopic analysis of infected livers revealed that hepatic macrophages were generally infected with low (one to three) numbers of organisms, and that bacterial replication correlated with an increase in the number of infected macrophages and foci of infection, rather than replication within already infected cells or expansion of existing foci [105]. The heterogeneity and stochastic nature of infection has been investigated in more detail using signature tags to distinguish bacteria. Tags were incorporated at the same chromosomal location without affecting virulence,

so that the resulting strain collection was phenotypically wild-type [106]. Eight of these wild-type isogenic tagged strains (WITS) were co-inoculated intravenously into various mouse strains and the bacteria recovered from the spleen, liver, MLNs, kidneys, heart and lungs analyzed to determine the dynamics of growth and killing of bacteria within an inoculum, and the contribution of certain host factors to this process. Thus it was shown that early in infection, the antagonistic processes of bacterial replication and killing by the oxidative burst gives rise to segregated, independent subpopulations of bacteria within different organs. Local expansion of these subpopulations ensues, with the subsequent detection of mixed populations at late stages of infection, coinciding with the presence of bacteria in the blood [106].

7.5 Perspectives

The enormous versatility and ease of use of the mouse model of salmonellosis has led to its intensive use over the last 20 years. It has provided a detailed understanding of much of the natural history of the infectious process, the bacterial virulence genes involved and some of their functions, as well as the host resistance mechanisms that arise during infection. Despite this, many important questions remain. We know that many of the interactions are stochastic, but we do not know the mechanisms underlying this, or why some interactions result in pathogen death whereas others lead to pathogen growth. We do not yet have the imaging capability to follow bacterial cells from invasion of Peyer's Patches through to interactions with DCs in MLNs and on to macrophages in the spleen and liver. We lack an understanding of the factors that underlie the establishment of chronic infection, the specific physiological targets for many type III effector proteins, and how all of these contribute to the avoidance of killing and bacterial replication. Nevertheless, with the development of more advanced imaging, genetic and biochemical techniques, we can anticipate a fuller understanding of the spatiotemporal development of systemic infection and the host–pathogen interactions that take place at a tissue, cellular and molecular level. The applicability of what we have learnt from the murine typhoid model to human infection by *S. typhi* has already been demonstrated: for example, an orally-delivered single dose of an attenuated strain carrying mutations in *ssaV* and *aroA* shows considerable promise as a new rationally designed typhoid vaccine [52]. Further studies on infection of mice by *S. typhimurium* promise to provide a more thorough understanding of host resistance, some of which may well prove relevant in the broader context of bacterial infection. This will undoubtedly aid our future approaches to vaccine design, as well as exposing aspects of virulence that could be targeted by small molecules to reduce and possibly cure infection.

References

1 Crump, J.A., Luby, S.P., and Mintz, E.D. (2004) The global burden of typhoid fever. *Bull. World Health Organ.*, **82**, 346–353.

2 Pang, T., Levine, M.M., Ivanoff, B., Wain, J., and Finlay, B.B. (1998) Typhoid fever–important issues still remain. *Trends Microbiol.*, **6**, 131–133.

3 Bhan, M.K., Bahl, R., and Bhatnagar, S. (2005) Typhoid and paratyphoid fever. *Lancet*, **366**, 749–762.

4 Khan, S.A., Stratford, R., Wu, T., McKelvie, N., Bellaby, T., Hindle, Z., Sinha, K.A., Eltze, S., Mastroeni, P., Pickard, D., Dougan, G., Chatfield, S.N., and Brennan, F.R. (2003) *Salmonella typhi* and *S. typhimurium* derivatives harbouring deletions in aromatic biosynthesis and Salmonella Pathogenicity Island-2 (SPI-2) genes as vaccines and vectors. *Vaccine*, **21**, 538–548.

5 House, D., Bishop, A., Parry, C., Dougan, G., and Wain, J. (2001) Typhoid fever: pathogenesis and disease. *Curr. Opin. Infect. Dis.*, **14**, 573–578.

6 Clark, M.A., Jepson, M.A., Simmons, N.L., and Hirst, B.H. (1994) Preferential interaction of Salmonella typhimurium with mouse Peyer's patch M cells. *Res. Microbiol.*, **145**, 543–552.

7 Clark, M.A., Reed, K.A., Lodge, J., Stephen, J., Hirst, B.H., and Jepson, M.A. (1996) Invasion of murine intestinal M cells by *Salmonella typhimurium inv* mutants severely deficient for invasion of cultured cells. *Infect. Immun.*, **64**, 4363–4368.

8 Jones, B.D., Ghori, N., and Falkow, S. (1994) *Salmonella typhimurium* initiates murine infection by penetrating and destroying the specialized epithelial M cells of the Peyer's patches. *J. Exp. Med.*, **180**, 15–23.

9 Vazquez-Torres, A., Jones-Carson, J., Baumler, A.J., Falkow, S., Valdivia, R., Brown, W., Le, M., Berggren, R., Parks, W.T., and Fang, F.C. (1999) Extraintestinal dissemination of Salmonella by CD18-expressing phagocytes. *Nature*, **401**, 804–808.

10 Carter, P.B., and Collins, F.M. (1974) The route of enteric infection in normal mice. *J. Exp. Med.*, **139**, 1189–1203.

11 Monack, D.M., Bouley, D.M., and Falkow, S. (2004) *Salmonella typhimurium* persists within macrophages in the mesenteric lymph nodes of chronically infected Nramp1+/+ mice and can be reactivated by IFNgamma neutralization. *J. Exp. Med.*, **199**, 231–241.

12 Porwollik, S., Boyd, E.F., Choy, C., Cheng, P., Florea, L., Proctor, E., and McClelland, M. (2004) Characterization of *Salmonella enterica* subspecies I genovars by use of microarrays. *J. Bacteriol.*, **186**, 5883–5898.

13 van Velkinburgh, J.C., and Gunn, J.S. (1999) PhoP-PhoQ-regulated loci are required for enhanced bile resistance in Salmonella spp. *Infect. Immun.*, **67**, 1614–1622.

14 McClelland, M., Sanderson, K.E., Spieth, J., Clifton, S.W., Latreille, P., Courtney, L., Porwollik, S., Ali, J., Dante, M., Du, F., Hou, S., Layman, D., Leonard, S., Nguyen, C., Scott, K., Holmes, A., Grewal, N., Mulvaney, E., Ryan, E., Sun, H., Florea, L., Miller, W., Stoneking, T., Nhan, M., Waterston, R., and Wilson, R.K. (2001) Complete genome sequence of *Salmonella enterica* serovar Typhimurium LT2. *Nature*, **413**, 852–856.

15 Parkhill, J., Dougan, G., James, K.D., Thomson, N.R., Pickard, D., Wain, J., Churcher, C., Mungall, K.L., Bentley, S.D., Holden, M.T., Sebaihia, M., Baker, S., Basham, D., Brooks, K., Chillingworth, T., Connerton, P., Cronin, A., Davis, P., Davies, R.M., Dowd, L., White, N., Farrar, J., Feltwell, T., Hamlin, N., Haque, A., Hien, T.T., Holroyd, S., Jagels, K., Krogh, A., Larsen, T.S., Leather, S., Moule, S., O'Gaora, P., Parry, C., Quail, M., Rutherford, K., Simmonds, M., Skelton, J., Stevens, K., Whitehead, S., and Barrell, B.G. (2001) Complete genome sequence of a multiple drug resistant *Salmonella enterica* serovar Typhi CT18. *Nature*, **413**, 848–852.

16 Hensel, M. (2004) Evolution of pathogenicity islands of *Salmonella enterica*. *Int. J. Med. Microbiol.*, **294**, 95–102.

17 Ochman, H., and Groisman, E.A. (1996) Distribution of pathogenicity islands in Salmonella spp. *Infect. Immun.*, **64**, 5410–5412.

18 Townsend, S.M., Kramer, N.E., Edwards, R., Baker, S., Hamlin, N., Simmonds, M., Stevens, K., Maloy, S., Parkhill, J., Dougan, G., and Baumler, A.J. (2001) *Salmonella enterica* serovar Typhi possesses a unique repertoire of fimbrial gene sequences. *Infect. Immun.*, **69**, 2894–2901.

19 Boyd, E.F., and Hartl, D.L. (1998) Salmonella virulence plasmid. Modular acquisition of the *spv* virulence region by an F-plasmid in *Salmonella enterica* subspecies I and insertion into the chromosome of subspecies II, IIIa, IV and VII isolates. *Genetics*, **149**, 1183–1190.

20 Hapfelmeier, S., Stecher, B., Barthel, M., Kremer, M., Muller, A.J., Heikenwalder, M., Stallmach, T., Hensel, M., Pfeffer, K., Akira, S., and Hardt, W.D. (2005) The Salmonella pathogenicity island (SPI)-2 and SPI-1 type III secretion systems allow Salmonella serovar typhimurium to trigger colitis via MyD88-dependent and MyD88-independent mechanisms. *J. Immunol.*, **174**, 1675–1685.

21 Miller, C.P., and Bohnhoff, M. (1963) Changes in the mouse's enteric microflora associated with enhanced susceptibility to salmonella infection following streptomycin treatment. *J. Infect. Dis.*, **113**, 59–66.

22 Fields, P.I., Swanson, R.V., Haidaris, C.G., and Heffron, F. (1986) Mutants of *Salmonella typhimurium* that cannot survive within the macrophage are avirulent. *Proc. Natl Acad. Sci. USA*, **83**, 5189–5193.

23 Fields, P.I., Groisman, E.A., and Heffron, F. (1989) A Salmonella locus that controls resistance to microbicidal proteins from phagocytic cells. *Science*, **243**, 1059–1062.

24 Galan, J.E., and Curtiss, R., 3rd (1989) Cloning and molecular characterization of genes whose products allow Salmonella typhimurium to penetrate tissue culture cells. *Proc. Natl Acad. Sci. USA*, **86**, 6383–6387.

25 Groisman, E.A., Chiao, E., Lipps, C.J., and Heffron, F. (1989) *Salmonella typhimurium phoP* virulence gene is a transcriptional regulator. *Proc. Natl Acad. Sci. USA*, **86**, 7077–7081.

26 Miller, S.I., Kukral, A.M., and Mekalanos, J.J. (1989) A two-component regulatory system (phoP phoQ) controls *Salmonella typhimurium* virulence. *Proc. Natl Acad. Sci. USA*, **86**, 5054–5058.

27 Hsu, H.S. (1989) Pathogenesis and immunity in murine salmonellosis. *Microbiol. Rev.*, **53**, 390–409.

28 Mahan, M.J., Tobias, J.W., Slauch, J.M., Hanna, P.C., Collier, R.J., and Mekalanos, J.J. (1995) Antibiotic-based selection for bacterial genes that are specifically induced during infection of a host. *Proc. Natl Acad. Sci. USA*, **92**, 669–673.

29 Camilli, A., Beattie, D.T., and Mekalanos, J.J. (1994) Use of genetic recombination as a reporter of gene expression. *Proc. Natl Acad. Sci. USA*, **91**, 2634–2638.

30 Hensel, M., Shea, J.E., Gleeson, C., Jones, M.D., Dalton, E., and Holden, D.W. (1995) Simultaneous identification of bacterial virulence genes by negative selection. *Science*, **269**, 400–403.

31 Zbell, A.L., Maier, S.E., and Maier, R.J. (2008) *Salmonella enterica* serovar Typhimurium NiFe uptake-type hydrogenases are differentially expressed *in vivo*. *Infect. Immun.*, **76**, 4445–4454.

32 Brown, N.F., Vallance, B.A., Coombes, B.K., Valdez, Y., Coburn, B.A., and Finlay, B.B. (2005) Salmonella pathogenicity island 2 is expressed prior to penetrating the intestine. *PLoS Pathog.*, **1**, e32.

33 Valdivia, R.H., and Falkow, S. (1997) Fluorescence-based isolation of bacterial genes expressed within host cells. *Science*, **277**, 2007–2011.

34 Cirillo, D.M., Valdivia, R.H., Monack, D.M., and Falkow, S. (1998) Macrophage-dependent induction of the Salmonella pathogenicity island 2 type

III secretion system and its role in intracellular survival. *Mol. Microbiol.*, **30**, 175–188.

35 Salcedo, S.P., Noursadeghi, M., Cohen, J., and Holden, D.W. (2001) Intracellular replication of *Salmonella typhimurium* strains in specific subsets of splenic macrophages *in vivo*. *Cell. Microbiol.*, **3**, 587–597.

36 Mazurkiewicz, P., Tang, C.M., Boone, C., and Holden, D.W. (2006) Signature-tagged mutagenesis: barcoding mutants for genome-wide screens. *Nat. Rev. Genet.*, **7**, 929–939.

37 Shea, J.E., Hensel, M., Gleeson, C., and Holden, D.W. (1996) Identification of a virulence locus encoding a second type III secretion system in *Salmonella typhimurium*. *Proc. Natl Acad. Sci. USA*, **93**, 2593–2597.

38 Ochman, H., Soncini, F.C., Solomon, F., and Groisman, E.A. (1996) Identification of a pathogenicity island required for Salmonella survival in host cells. *Proc. Natl Acad. Sci. USA*, **93**, 7800–7804.

39 Shea, J.E., Beuzon, C.R., Gleeson, C., Mundy, R., and Holden, D.W. (1999) Influence of the *Salmonella typhimurium* pathogenicity island 2 type III secretion system on bacterial growth in the mouse. *Infect. Immun.*, **67**, 213–219.

40 Vazquez-Torres, A., Xu, Y., Jones-Carson, J., Holden, D.W., Lucia, S.M., Dinauer, M.C., Mastroeni, P., and Fang, F.C. (2000) Salmonella pathogenicity island 2-dependent evasion of the phagocyte NADPH oxidase. *Science*, **287**, 1655–1658.

41 Chakravortty, D., Hansen-Wester, I., and Hensel, M. (2002) Salmonella pathogenicity island 2 mediates protection of intracellular Salmonella from reactive nitrogen intermediates. *J. Exp. Med.*, **195**, 1155–1166.

42 Cheminay, C., Mohlenbrink, A., and Hensel, M. (2005) Intracellular Salmonella inhibit antigen presentation by dendritic cells. *J. Immunol.*, **174**, 2892–2899.

43 Mazurkiewicz, P., Thomas, J., Thompson, J.A., Liu, M., Arbibe, L., Sansonetti, P., and Holden, D.W. (2008) SpvC is a Salmonella effector with phosphothreonine lyase activity on host mitogen-activated protein kinases. *Mol. Microbiol.*, **67**, 1371–1383.

44 Mitchell, E.K., Mastroeni, P., Kelly, A.P., and Trowsdale, J. (2004) Inhibition of cell surface MHC class II expression by Salmonella. *Eur. J. Immunol.*, **34**, 2559–2567.

45 Boucrot, E., Beuzon, C.R., Holden, D.W., Gorvel, J.P., and Meresse, S. (2003) *Salmonella typhimurium* SifA effector protein requires its membrane-anchoring C-terminal hexapeptide for its biological function. *J. Biol. Chem.*, **278**, 14196–14202.

46 Henry, T., Couillault, C., Rockenfeller, P., Boucrot, E., Dumont, A., Schroeder, N., Hermant, A., Knodler, L.A., Lecine, P., Steele-Mortimer, O., Borg, J.P., Gorvel, J.P., and Meresse, S. (2006) The Salmonella effector protein PipB2 is a linker for kinesin-1. *Proc. Natl Acad. Sci. USA*, **103**, 13497–13502.

47 Ruiz-Albert, J., Yu, X.J., Beuzon, C.R., Blakey, A.N., Galyov, E.E., and Holden, D.W. (2002) Complementary activities of SseJ and SifA regulate dynamics of the *Salmonella typhimurium* vacuolar membrane. *Mol. Microbiol.*, **44**, 645–661.

48 Coombes, B.K., Lowden, M.J., Bishop, J.L., Wickham, M.E., Brown, N.F., Duong, N., Osborne, S., Gal-Mor, O., and Finlay, B.B. (2007) SseL is a salmonella-specific translocated effector integrated into the SsrB-controlled salmonella pathogenicity island 2 type III secretion system. *Infect. Immun.*, **75**, 574–580.

49 Le Negrate, G., Faustin, B., Welsh, K., Loeffler, M., Krajewska, M., Hasegawa, P., Mukherjee, S., Orth, K., Krajewski, S., Godzik, A., Guiney, D.G., and Reed, J.C. (2008) Salmonella secreted factor L deubiquitinase of *Salmonella typhimurium* inhibits NF-kappaB, suppresses IkappaBalpha ubiquitination and modulates innate immune responses. *J. Immunol.*, **180**, 5045–5056.

50 Rytkonen, A., Poh, J., Garmendia, J., Boyle, C., Thompson, A., Liu, M., Freemont, P., Hinton, J.C., and Holden, D.W. (2007) SseL, a Salmonella deubiquitinase required for

macrophage killing and virulence. *Proc. Natl Acad. Sci. USA*, **104**, 3502–3507.

51 Worley, M.J., Nieman, G.S., Geddes, K., and Heffron, F. (2006) *Salmonella typhimurium* disseminates within its host by manipulating the motility of infected cells. *Proc. Natl Acad. Sci. USA*, **103**, 17915–17920.

52 Kirkpatrick, B.D., McKenzie, R., O'Neill, J.P., Larsson, C.J., Bourgeois, A.L., Shimko, J., Bentley, M., Makin, J., Chatfield, S., Hindle, Z., Fidler, C., Robinson, B.E., Ventrone, C.H., Bansal, N., Carpenter, C.M., Kutzko, D., Hamlet, S., LaPointe, C., and Taylor, D.N. (2006) Evaluation of *Salmonella enterica* serovar Typhi (Ty2 aroC-ssaV-) M01ZH09, with a defined mutation in the Salmonella pathogenicity island 2, as a live, oral typhoid vaccine in human volunteers. *Vaccine*, **24**, 116–123.

53 Galan, J.E., and Curtiss, R., 3rd (1989) Virulence and vaccine potential of phoP mutants of *Salmonella typhimurium*. *Microb. Pathog.*, **6**, 433–443.

54 Gulig, P.A., Danbara, H., Guiney, D.G., Lax, A.J., Norel, F., and Rhen, M. (1993) Molecular analysis of spv virulence genes of the Salmonella virulence plasmids. *Mol. Microbiol.*, **7**, 825–830.

55 Freter, R., Allweiss, B., O'Brien, P.C., Halstead, S.A., and Macsai, M.S. (1981) Role of chemotaxis in the association of motile bacteria with intestinal mucosa: *in vitro* studies. *Infect. Immun.*, **34**, 241–249.

56 Freter, R., O'Brien, P.C., and Macsai, M.S. (1981) Role of chemotaxis in the association of motile bacteria with intestinal mucosa: *in vivo* studies. *Infect. Immun.*, **34**, 234–240.

57 Becker, D., Selbach, M., Rollenhagen, C., Ballmaier, M., Meyer, T.F., Mann, M., and Bumann, D. (2006) Robust Salmonella metabolism limits possibilities for new antimicrobials. *Nature*, **440**, 303–307.

58 Crouch, M.L., Castor, M., Karlinsey, J.E., Kalhorn, T., and Fang, F.C. (2008) Biosynthesis and IroC-dependent export of the siderophore salmochelin are essential for virulence of *Salmonella enterica* serovar Typhimurium. *Mol. Microbiol.*, **67**, 971–983.

59 Gulig, P.A., and Doyle, T.J. (1993) The *Salmonella typhimurium* virulence plasmid increases the growth rate of salmonellae in mice. *Infect. Immun.*, **61**, 504–511.

60 Baumler, A.J., Tsolis, R.M., Valentine, P.J., Ficht, T.A., and Heffron, F. (1997) Synergistic effect of mutations in invA and lpfC on the ability of *Salmonella typhimurium* to cause murine typhoid. *Infect. Immun.*, **65**, 2254–2259.

61 Beuzon, C.R., and Holden, D.W. (2001) Use of mixed infections with Salmonella strains to study virulence genes and their interactions *in vivo*. *Microbes Infect.*, **3**, 1345–1352.

62 Beuzon, C.R., Unsworth, K.E., and Holden, D.W. (2001) *In vivo* genetic analysis indicates that PhoP-PhoQ and the Salmonella pathogenicity island 2 type III secretion system contribute independently to *Salmonella enterica* serovar Typhimurium virulence. *Infect. Immun.*, **69**, 7254–7261.

63 Mastroeni, P., Vazquez-Torres, A., Fang, F.C., Xu, Y., Khan, S., Hormaeche, C.E., and Dougan, G. (2000) Antimicrobial actions of the NADPH phagocyte oxidase and inducible nitric oxide synthase in experimental salmonellosis. II. Effects on microbial proliferation and host survival *in vivo*. *J. Exp. Med.*, **192**, 237–248.

64 Vazquez-Torres, A., Jones-Carson, J., Mastroeni, P., Ischiropoulos, H., and Fang, F.C. (2000) Antimicrobial actions of the NADPH phagocyte oxidase and inducible nitric oxide synthase in experimental salmonellosis. I. Effects on microbial killing by activated peritoneal macrophages in vitro. *J. Exp. Med.*, **192**, 227–236.

65 Vidal, S., Tremblay, M.L., Govoni, G., Gauthier, S., Sebastiani, G., Malo, D., Skamene, E., Olivier, M., Jothy, S., and Gros, P. (1995) The Ity/Lsh/Bcg locus: natural resistance to infection with intracellular parasites is abrogated by disruption of the Nramp1 gene. *J. Exp. Med.*, **182**, 655–666.

66 White, J.K., Mastroeni, P., Popoff, J.F., Evans, C.A., and Blackwell, J.M. (2005)

Slc11a1-mediated resistance to Salmonella enterica serovar Typhimurium and Leishmania donovani infections does not require functional inducible nitric oxide synthase or phagocyte oxidase activity. *J. Leukoc. Biol.*, **77**, 311–320.

67 Menager, N., Foster, G., Ugrinovic, S., Uppington, H., Verbeek, S., and Mastroeni, P. (2007) Fcgamma receptors are crucial for the expression of acquired resistance to virulent Salmonella enterica serovar Typhimurium in vivo but are not required for the induction of humoral or T-cell-mediated immunity. *Immunology*, **120**, 424–432.

68 Ugrinovic, S., Menager, N., Goh, N., Mastroeni, P. (2003) Characterization and development of T-Cell immune responses in B-cell-deficient (Igh-6(-/-)) mice with Salmonella enterica serovar Typhimurium infection. *Infect. Immun.*, **71**, 6808–6819.

69 Mittrucker, H.W., Raupach, B., Kohler, A., and Kaufmann, S.H. (2000) Cutting edge: role of B lymphocytes in protective immunity against Salmonella typhimurium infection. *J. Immunol.*, **164**, 1648–1652.

70 Warren, J., Mastroeni, P., Dougan, G., Noursadeghi, M., Cohen, J., Walport, M.J., and Botto, M. (2002) Increased susceptibility of C1q-deficient mice to Salmonella enterica serovar Typhimurium infection. *Infect. Immun.*, **70**, 551–557.

71 Raupach, B., Peuschel, S.K., Monack, D.M., and Zychlinsky, A. (2006) Caspase-1-mediated activation of interleukin-1beta (IL-1beta) and IL-18 contributes to innate immune defenses against Salmonella enterica serovar Typhimurium infection. *Infect. Immun.*, **74**, 4922–4926.

72 Bernheiden, M., Heinrich, J.M., Minigo, G., Schutt, C., Stelter, F., Freeman, M., Golenbock, D., and Jack, R.S. (2001) LBP, CD14, TLR4 and the murine innate immune response to a peritoneal Salmonella infection. *J. Endotoxin Res.*, **7**, 447–450.

73 Jack, R.S., Fan, X., Bernheiden, M., Rune, G., Ehlers, M., Weber, A., Kirsch, G., Mentel, R., Furll, B., Freudenberg, M., Schmitz, G., Stelter, F., and Schutt, C. (1997) Lipopolysaccharide-binding protein is required to combat a murine gram-negative bacterial infection. *Nature*, **389**, 742–745.

74 Vazquez-Torres, A., Vallance, B.A., Bergman, M.A., Finlay, B.B., Cookson, B.T., Jones-Carson, J., and Fang, F.C. (2004) Toll-like receptor 4 dependence of innate and adaptive immunity to Salmonella: importance of the Kupffer cell network. *J. Immunol.*, **172**, 6202–6208.

75 Weiss, D.S., Raupach, B., Takeda, K., Akira, S., and Zychlinsky, A. (2004) Toll-like receptors are temporally involved in host defense. *J. Immunol.*, **172**, 4463–4469.

76 Uematsu, S., Jang, M.H., Chevrier, N., Guo, Z., Kumagai, Y., Yamamoto, M., Kato, H., Sougawa, N., Matsui, H., Kuwata, H., Hemmi, H., Coban, C., Kawai, T., Ishii, K.J., Takeuchi, O., Miyasaka, M., Takeda, K., and Akira, S. (2006) Detection of pathogenic intestinal bacteria by Toll-like receptor 5 on intertinal CD11c+ lamina propria cells. *Nat. Immunol.*, **7**, 868–874.

77 Henry, S.C., Daniell, X., Indaram, M., Whitesides, J.F., Sempowski, G.D., Howell, D., Oliver, T., and Taylor, G.A. (2007) Impaired macrophage function underscores susceptibility to Salmonella in mice lacking lrgm1 (LRG-47). *J. Immunol.* **179**, 6963–6972.

78 Fernandez-Cabezudo, M.J., Ullah, A., Flavell, R.A., and Al-Ramadi, B.K. (2005) Evidence for the requirement for CD40-CD154 interactions in resistance to infections with attenuated Salmonella. *J. Endotoxin Res.*, **11**, 395–399.

79 Ravindran, R., Foley, J., Stoklasek, T., Glimcher, L.H., and McSorley, S.J. (2005) Expression of T-bet by CD4 T cells is essential for resistance to Salmonella infection. *J. Immunol.*, **175**, 4603–4610.

80 Kim, K.I., Malakhova, O.A., Hoebe, K., Yan, M., Beutler, B., and Zhang, D.E. (2005) Enhanced antibacterial potential in UBP43-deficient mice against Salmonella typhimurium infection by

up-regulating type I IFN signaling. *J. Immunol.*, **175**, 847–854.

81 Medina, F.A., de Almeida, C.J., Dew, E., Li, J., Bonuccelli, G., Williams, T.M., Cohen, A.W., Pestell, R.G., Frank, P.G., Tanowitz, H.B., and Lisanti, M.P. (2006) Caveolin-1-deficient mice show defects in innate immunity and inflammatory immune response during Salmonella enterica serovar Typhimurium infection. *Infect. Immun.*, **74**, 6665–6674.

82 Everest, P., Roberts, M., and Dougan, G. (1998) Susceptibility to Salmonella typhimurium infection and effectiveness of vaccination in mice deficient in the tumor necrosis factor alpha p55 receptor. *Infect. Immun.*, **66**, 3355–3364.

83 Everest, P., Allen, J., Papakonstantinopoulou, A., Mastroeni, P., Roberts, M., and Dougan, G. (1997) Salmonella typhimurium infections in mice deficient in interleukin-4 production: role of IL-4 in infection-associated pathology. *J. Immunol.*, **159**, 1820–1827.

84 Conlan, J.W. (1997) Critical roles of neutrophils in host defense against experimental systemic infections of mice by *Listeria monocytogenes*, *Salmonella typhimurium*, and *Yersinia enterocolitica*. *Infect. Immun.*, **65**, 630–635.

85 Mastroeni, P., Harrison, J.A., Chabalgoity, J.A., and Hormaeche, C.E. (1996) Effect of interleukin 12 neutralization on host resistance and gamma interferon production in mouse typhoid. *Infect. Immun.*, **64**, 189–196.

86 Dybing, J.K., Walters, N., and Pascual, D.W. (1999) Role of endogenous interleukin-18 in resolving wild-type and attenuated *Salmonella typhimurium* infections. *Infect. Immun.*, **67**, 6242–6248.

87 Mastroeni, P., Clare, S., Khan, S., Harrison, J.A., Hormaeche, C.E., Okamura, H., Kurimoto, M., and Dougan, G. (1999) Interleukin 18 contributes to host resistance and gamma interferon production in mice infected with virulent *Salmonella typhimurium*. *Infect. Immun.*, **67**, 478–483.

88 Govoni, G., Canonne-Hergaux, F., Pfeifer, C.G., Marcus, S.L., Mills, S.D., Hackam, D.J., Grinstein, S., Malo, D., Finlay, B.B., and Gros, P. (1999) Functional expression of Nramp1 *in vitro* in the murine macrophage line RAW264.7. *Infect. Immun.*, **67**, 2225–2232.

89 Valdez, Y., Diehl, G.E., Vallance, B.A., Grassl, G.A., Guttman, J.A., Brown, N.F., Rosenberger, C.M., Littman, D.R., Gros, P., and Finlay, B.B. (2008) Nramp1 expression by dendritic cells modulates inflammatory responses during *Salmonella typhimurium* infection. *Cell. Microbiol.*, **10**, 1646–1661.

90 Valdez, Y., Grassl, G.A., Guttman, J.A., Coburn, B., Gros, P., Vallance, B.A., and Finlay, B.B. (2009) Nramp1 drives an accelerated inflammatory response during Salmonella-induced colitis in mice. *Cell. Microbiol.*, **11**, 351–362.

91 Chieppa, M., Rescigno, M., Huang, A.Y., and Germain, R.N. (2006) Dynamic imaging of dendritic cell extension into the small bowel lumen in response to epithelial cell TLR engagement. *J. Exp. Med.*, **203**, 2841–2852.

92 Biedzka-Sarek, M., and El Skurnik, M. (2006) How to outwit the enemy: dendritic cells face Salmonella. *APMIS*, **114**, 589–600.

93 Iwasaki, A., and Kelsall, B.L. (2000) Localization of distinct Peyer's patch dendritic cell subsets and their recruitment by chemokines macrophage inflammatory protein (MIP)-3alpha, MIP-3beta, and secondary lymphoid organ chemokine. *J. Exp. Med.*, **191**, 1381–1394.

94 Rescigno, M., Urbano, M., Valzasina, B., Francolini, M., Rotta, G., Bonasio, R., Granucci, F., Kraehenbuhl, J.P., and Ricciardi-Castagnoli, P. (2001) Dendritic cells express tight junction proteins and penetrate gut epithelial monolayers to sample bacteria. *Nat. Immunol.*, **2**, 361–367.

95 Hisert, K.B., Kirksey, M.A., Gomez, J.E., Sousa, A.O., Cox, J.S., Jacobs, W.R., Jr., Nathan, C.F., and McKinney, J.D. (2004) Identification of *Mycobacterium tuberculosis* counterimmune (cim)

mutants in immunodeficient mice by differential screening. *Infect. Immun.*, **72**, 5315–5321.

96 Hisert, K.B., MacCoss, M., Shiloh, M.U., Darwin, K.H., Singh, S., Jones, R.A., Ehrt, S., Zhang, Z., Gaffney, B.L., Gandotra, S., Holden, D.W., Murray, D., and Nathan, C. (2005) A glutamate-alanine-leucine (EAL) domain protein of Salmonella controls bacterial survival in mice, antioxidant defence and killing of macrophages: role of cyclic diGMP. *Mol. Microbiol.*, **56**, 1234–1245.

97 Martinez-Argudo, I., and Jepson, M.A. (2008) Salmonella translocates across an *in vitro* M cell model independently of SPI-1 and SPI-2. *Microbiology*, **154**, 3887–3894.

98 Contag, C.H., Contag, P.R., Mullins, J.I., Spilman, S.D., Stevenson, D.K., and Benaron, D.A. (1995) Photonic detection of bacterial pathogens in living hosts. *Mol. Microbiol.*, **18**, 593–603.

99 Richter-Dahlfors, A., Buchan, A.M., and Finlay, B.B. (1997) Murine salmonellosis studied by confocal microscopy: Salmonella typhimurium resides intracellularly inside macrophages and exerts a cytotoxic effect on phagocytes *in vivo*. *J. Exp. Med.*, **186**, 569–580.

100 Hopkins, S.A., Niedergang, F., Corthesy-Theulaz, I.E., and Kraehenbuhl, J.P. (2000) A recombinant *Salmonella typhimurium* vaccine strain is taken up and survives within murine Peyer's patch dendritic cells. *Cell. Microbiol.*, **2**, 59–68.

101 Beuzon, C.R., Meresse, S., Unsworth, K.E., Ruiz-Albert, J., Garvis, S., Waterman, S.R., Ryder, T.A., Boucrot, E., and Holden, D.W. (2000) Salmonella maintains the integrity of its intracellular vacuole through the action of SifA. *EMBO J.*, **19**, 3235–3249.

102 Bumann, D. (2002) Examination of Salmonella gene expression in an infected mammalian host using the green fluorescent protein and two-colour flow cytometry. *Mol. Microbiol.*, **43**, 1269–1283.

103 Thone, F., Schwanhausser, B., Becker, D., Ballmaier, M., and Bumann, D. (2007) FACS-isolation of Salmonella-infected cells with defined bacterial load from mouse spleen. *J. Microbiol. Methods*, **71**, 220–224.

104 Geddes, K., Cruz, F., and Heffron, F. (2007) Analysis of cells targeted by salmonella type III secretion *in vivo*. *PLoS Pathog.*, **3**, e196.

105 Sheppard, M., Webb, C., Heath, F., Mallows, V., Emilianus, R., Maskell, D., and Mastroeni, P. (2003) Dynamics of bacterial growth and distribution within the liver during Salmonella infection. *Cell. Microbiol.*, **5**, 593–600.

106 Grant, A.J., Restif, O., McKinley, T.J., Sheppard, M., Maskell, D.J., and Mastroeni, P. (2008) Modelling within-host spatiotemporal dynamics of invasive bacterial disease. *PLoS Biol.*, **6**, e74.

107 Meynell, G.G. and Stocker, B.A. (1957) Some hypotheses on the aetiology of fatal infections in partially resistant hosts and their application to mice challenged with Salmonella paratyphi-B or Salmonella typhimurium by intraperitoneal injection. *J. Gen. Microbiol.*, **16**, 38–58.

8
Chlamydia: from Molecular Insight to Therapeutic Discovery

Lesley A. Ogilvie, Dagmar Heuer, and Thomas F. Meyer

8.1
Introduction

Intracellular pathogens present a unique state of affairs when it comes to the study and treatment (or prevention) of the ills they cause. Hidden in the cell, often within a protective niche, the intracellular dweller must obtain essential nutrients for replication and escape detection by the host immune system and its catalog of defense mechanisms. Microbes of socio-economic and medical importance causing diseases such as tuberculosis, typhus, pneumonia, trachoma, gastroenteritis and sexually transmitted infections often have an intracellular stage of existence. This intimate and unique association of host and pathogen has driven a co-evolution of defense, detection, evasion and procurement strategies that are now the subject of an intense research effort.

Approaches to the investigation of intracellular pathogens have faced a number of hurdles; however, the dawn of the post genomic era has heralded hope of new insight into the host–pathogen dynamic. To date, a representative genome of most, if not all, known human pathogens has been sequenced; in combination with human host genomic data, the foundations for discovery and insight have been laid. The development of global approaches to the investigation of gene function, including proteomics and transcriptomics as well as comparative genomics, are capitalizing on these ever burgeoning sequence banks and providing a means of eavesdropping on the cellular tête-à-tête between host and pathogen. But a major drawback exists: function is inferred not demonstrated. A loss-of-function approach to the global understanding of bacterial gene function has been provided by techniques such as transposon mutagenesis, but some bacteria, including *Chlamydia*, are recalcitrant to genetic manipulation, i.e. the ability to characterize the function and localization of a protein via the stable insertion and integration of heterologous DNA into the genome is lacking. In the absence of genetic methodologies, a host-oriented approach to the investigation of pathogen–host interactions may prove to be more fruitful. This era of a global appreciation of host responses to pathogen invasion is now upon us. Great strides in understanding have already been made

Bacterial Virulence: Basic Principles, Models and Global Approaches. Edited by Philippe Sansonetti
© 2010 WILEY-VCH Verlag GmbH & Co. KGaA, Weinheim
ISBN: 978-3-527-32326-5

through the use of knock-out mice to elucidate host gene function. These infection models have aided the study of gene function in a range of biological processes and aided the discovery of new therapeutics [1]; however, the generation time, numbers of animals required to provide statistically relevant data, a lack of robust phenotypes, plus other practical concerns represent a bottleneck to the rapid elucidation of gene function and the discovery of new therapeutics. Recent progress in the development of novel global approaches, such as RNA interference (RNAi), which allow the analysis of host cell function and are amenable to both high-throughput and *in vivo* applications, are providing unprecedented access to the host–pathogen interface. The identification of host cell factors crucial for each stage of the development cycle and therefore successful pathogenesis is now a realistic goal.

To date, data generated has uncovered an unexpected subtlety, complexity and dynamism in the host–pathogen interplay; the extent to which intracellular pathogens can manipulate the host cell cytoskeleton to efficiently adhere and enter host cells, procure host cell constituents to fashion a protective niche (e.g. pathogens such as *Chlamydia*, *Salmonella, Mycobacterium* and *Legionella* multiply within a protective membrane bound vacuole, whilst others such as *Shigella* and *Listeria* are able to replicate within the host cell cytosol), escape detection by the host immune system, subvert host cell signaling pathways to acquire essential nutrients [2, 3] and make their exit, ready and able to infect new cells or hosts [4]. These studies are revealing the importance of host factors in the pathogenesis of infection, with the recognition of the impact of genetic predisposition on the susceptibility to and severity of infection. Correspondingly, the key role bacterial factors such as adhesions, pathogen-associated molecular patterns (PAMPS), other effector proteins (bound and secreted) and bacterial systems, e.g. type III and IV secretory systems, play in altering host cell processes are also being elucidated.

It is the translation of this basic research into real-life therapeutic solutions that remains the current challenge in the continuing global struggle to control infectious diseases. For many pathogens available therapeutics are inadequate; resistance to antibiotics is increasing and at the same time discovery of novel antimicrobials is declining; therefore, alternative sources of therapeutics are urgently required. The novel concept of host cell factor-oriented therapeutics may well represent a new frontier in the treatment of infectious diseases.

Chlamydia are intracellular pathogens causing a range of diseases with major socio-economic significance. Since these bacteria present a particular challenge for investigating the intimate association with their host from the bacterial perspective, they represent an ideal model to test a host-focused global approach to identify the molecular and cellular basis for pathogenesis and/or host pathogen interactions. Integration of knowledge gained may provide the opportunity to interfere with the onset, development and persistency of chlamydial infection and pave the way for the development of novel diagnostic and therapeutic treatment regimes. Focusing on *C. trachomatis*, an important human intracellular pathogen, here we report on the current state of research, emphasizing the key insights that are resulting from a host-oriented global analysis of *Chlamydia*–host interactions.

8.2 *Chlamydia*: a Model Intracellular Pathogen–Host Relationship

Since chlamydial infection was first detected in 1907 by Halberstaedter and von Prowazek [5], these Gram negative, obligate intracellular bacterial pathogens of humans and animals have been shown to be the etiological agents of a range of acute and chronic diseases with significant socio-economic and medical impact. The main species that are important in human infections are *C. trachomatis* and *C. pneumoniae*.

C. trachomatis infects genital and ocular epithelial cells, leading to diseases such as urethritis, salpingitis and conjunctivitis. Based on the differential immunoreactivity of the major outer membrane protein (MOMP) encoded by *ompA*, *C. trachomatis* is grouped into different serotypes: serotypes A, B, Ba and C are generally found in ocular epithelia whereas serotypes D–K affect epithelial surfaces of the genital tract [6]. Serovars L1, L2 and L3 can disseminate and infect monocytes in lymph nodes to cause lymphogranuloma venereum [7]. Polymorphism of the pathogen's tryptophan synthase (*trpBA*) genes can also dictate tissue tropism [8].Chronic recurring infections lead to severe disease sequelae such as pelvic inflammatory disease (PID), sterility and ectopic pregnancy in women, and the preventable blindness, trachoma. The World Health Organization (WHO) reports that there are currently 140 million people with *C. trachomatis* ocular infections worldwide and of those, 6 million people are blind. The global eradication of trachoma as a disease of public health importance is a WHO target for 2020. Annually, 4–5 million cases of sexually transmitted *C. trachomatis* infections are reported in the USA alone (www.who.int).

C. pneumoniae primarily infects the human respiratory tract and is the etiological agent of approximately 10% of community-acquired pneumonia and 5% of bronchitis, sinusitis and pharyngitis cases. Up to 50% of the population in the developed world are seropositive by the age of 20 years [9]. The bacterium is implicated in the development of Alzheimer's disease [10], atherosclerosis [11], arthritis [12] and chronic pulmonary disease [13]; however, the true nature of these associations is still not clear.

C. trachomatis and *C. pneumoniae* are members of the phylum Chlamydiae, a diverse group of pathogens with a seemingly ubiquitous distribution, from free-living amoebae to humans [14]. Novel Chlamydiae are still being discovered, most recently from a wastewater plant [15]. Some of these recent discoveries can be considered potential emerging pathogens: one such strain, *Simkania negevensis* [16] has been associated with child bronchitis [17] and also with community-acquired pneumonia in adults [18] and children [19]. A somewhat overlooked member of the Chlamydiae *C. psittaci* is normally an avian pathogen but is known to have zoonotic potential, causing severe pneumonia in humans [20]. Another relative, the murine pathogen *C. muridarum* is often used as a surrogate infection model for the human pathogen *C. trachomatis* due to striking homology in their genome sequences [21].

To date, the only effective cure for *Chlamydia* infections is long-term treatment with antibiotics such as azithromycin, doxycycline and tetracycline; however,

treatment failure and/or re-occurrence of infection are becoming more common. Numerous studies have reported a growing resistance against many classes of antibiotics, especially in *C. trachomatis*, e.g. [22–26]. Antibiotics may clear an acute infection of *Chlamydia*, but may drive the bacterium into a state of persistence [27–29] and indeed into a cycle of chronic infection. In addition, despite molecular analysis of bacterial components crucial for pathogen replication and survival which have identified promising novel targets for the development of new antibiotics, not a single new antimicrobial has entered late-stage clinical trials [30]. In addition, it has been suggested that the number of targets and pathways essential to pathogenesis that can be utilized for broad spectrum antibiotics have inherent limitations [31].

Molecular investigations are unearthing a variety of promising targets for use in vaccination, including chlamydial conserved proteins such as MOMP and the ribonucleotide reductase small chain protein (NrdB), which has been demonstrated to play a crucial role in *Chlamydia* replication [32, 33]; other targets include proteases [34] and EB subunits, used in combination with different delivery vehicles (e.g. recombinant proteins and DNA) and routes, all with varied success (see [35] for an in-depth review). Despite intense research effort, no effective vaccine against genital or ocular *Chlamydia* infection in humans has been developed.

8.2.1
The Cycle of Development – *C. trachomatis*

The growth of *Chlamydia* is inevitably bound to the function of its host cell. These bacteria have a unique biphasic lifestyle that alternates between two functionally and morphologically distinct forms, namely the infectious but metabolically inactive elementary body (EB) and the non-infectious but replicating reticulate body (RB) (see Figure 8.1). Although the chlamydial intracellular lifestyle and unique cycle of development hamper diagnosis, treatment and analysis of the infections these bacteria cause, studies to date are providing crucial insight into the key stages of the infection:

Figure 8.1 The *C. trachomatis* cycle of development. *Chlamydia* have a unique biphasic cycle of development that alternates between two functionally and morphologically distinct forms, namely the infectious but metabolically inactive elementary body (EB) and the non-infectious but replicating reticulate body (RB). The cross talk between *Chlamydia* and its host drives a cycle of development from initial attachment to host cell exit and persistence. The inactive but infectious EB attaches to the surface of host cells, enters and within the cell transforms into the non-infectious but replicating RB. The transition from EB to RB is accompanied by the development of a replicative niche, termed the inclusion. After 2–3 days, the RBs leave the cell ready and able to infect a new host or cell. During periods of severe nutrient limitation *Chlamydia* may also enter a state of persistence. Bacterial and host cell factors implicated in these different steps are shown here.

1. Attachment & invasion
Glycan
MOMP
GAG
OmcB
?
PDGFRβ
PDI
?
TARP
P
SFγ
Abl
Actin
Arp2/3
Rac
WAVE2/Abi1
2. Inclusion formation
Rab11
?
Ct147
?
Dynein
p150Glued
MT
Ct229
Rab4
3. Expansion & nutrient acquisition
PLA2
P
NFκB
Raf
P
BH3
RFX5
CPAF
IF
Actin
Rho
Lipid Droplet
Vamp
Ct441
ChlaDub
Lda3
IncA
Ct229
?
IncG
Rab4
Rabs
?
Rab-Effectors
14-3-3β
MVB
GA
4. Host cell exit
[Ca2+]
Proteases
N-WASP
Actin
MyosinII
Rho
5. IFNγ-induced persistency
[Tryptophan]
IDO
IFNγ
TrpRBA
Indole
Serine
[Tryptophan]

(1) Attachment and Invasion *C. trachomatis* invasion begins as an EB attaches to the host cell and coordinates its internalization using a type III secretion system (TTSS)-mediated reorganization of the host cytoskeleton [36]. One theory is that initial binding is dependent on a tri-molecular bridge between chlamydial glycosaminoglycan (GAG) adhesin ligands and GAG receptors on the host cell and chlamydial outer membrane [37, 38]. This bridge may be supported by OmcB, a chlamydial surface-exposed protein that has been demonstrated to bind heparan sulfate-like GAGs [39, 40]; other factors such as the major outer membrane protein [41] and the protein disulfide isomerase [42] are also thought to play a role. *C. trachomatis* has a wide cell tropism, suggesting that there are multiple methods of entry into host cells. An elegant series of publications by Hackstadt and co-workers has shed some light on these means of entry [43–46]. Using a combination of live-cell imaging and immunofluorescence techniques, Translocated actin recruiting phosphoprotein (TARP), Wiskott-Aldridge syndrome protein family member 2 (WASP2) and Ras related protein 2 (Rac2) were shown to co-localize with EBs at the site of entry [45, 46]. Infection results in Rac activation, promoting an interaction with WAVE2 and Abelson-interacting protein-1 (AbI-1) to activate the Arp2/3 complex, a seven subunit protein, thus facilitating invasion via the induction of actin cytoskeletal rearrangements [45]. Recently, using RNAi Elwell and co-workers identified PDGFRβ signaling as an important factor, but redundant with AbI kinase targeting the TARP actin nucleator, in the early stages of chlamydial infection [47]. TARP is thought to be a substrate of multiple kinases [48] including AbI kinase [47] and Src family kinases (SFK) [46].

(2) Inclusion Formation Within the first 2 h after infection, *Chlamydia* remodel host cell components to fashion their protective niche via the secretion of chlamydial effector proteins [49]. This membrane-bound vesicle, termed the inclusion, not only provides protection from host defenses by circumventing endosomal and lysosymal pathways [50–52] and allowing the bacterium to escape host cell apoptosis [53, 54], but also allows exchange of bacterial effector molecules with the host cytoplasm; for instance, the chlamydial effector protein ct147 is transcribed early during the infection cycle and is post translationally modified [55], indicating that it plays a significant biological role. The inclusion (plus its associated subunit $p150^{(Glued)}$) is then transported in a dynein- and microtubule-dependent [56, 57] manner to the peri-nuclear region where the transition from the infectious EB to the replicating RB occurs. During this time a subset of Rab GTPases (including Rab 11 and Rab 4) are recruited to the inclusion membrane. These ubiquitous 'molecular switches', Rab GTPases plus their effectors, are thought to play a central role in regulating membrane transport and organelle identity [58].

(3) Expansion and Nutrient Acquisition The inclusion expands to accommodate the ever-increasing number progeny (approximately 1000 progeny per host cell) until it occupies most of the host cell cytoplasm. This expansion is likely fueled by the interception of vesicles from the Golgi apparatus [59–61], MVB-dependent pathways [62] and translocation of lipid droplets (LDs) and neutral lipid storage organelles from the host cytoplasm into the inclusion lumen [63] to provide essen-

tial lipids for growth. Some progress is being made in elucidating the mechanics of interception: the inclusion membrane proteins (Inc) [64] are thought to be integral to re-routing lipid traffic to the inclusion [49] and C. *trachomatis* Lda proteins are known to bind LDs, for example, Lda3 [63]. Also, activation of the host Raf-MEK-ERK-cPLA2 signaling cascade is required for uptake of host glycerophospholipids [65]. A more recent discovery by Heuer and co-workers, revealed that fragmentation of the Golgi apparatus into 'ministacks' increases replication and lipid acquisition [66]. From within the growing inclusion, chlamydial effector proteins orchestrate the evasion and disabling of host immune responses by subversion of NF-kB signaling and the degradation of host transcription factors and pro-apoptotic proteins (e.g. BH3 [67]). Both *Chl*Dub1 production [68] and the proteolytic cleavage of p65 (an Nf-kB subunit) by Ct441 [69] were shown to interfere with NF-κB activation. In addition, the chlamydial protease-like activity factor (CPAF) is secreted into the cytoplasm of the infected cells where it proteolytically cleaves the transcription factor RFX5 [70]. Translocation of CPAF into cytoplasm has been demonstrated to correlate in time with the degradation of RFX5 [71]. Other chlamydial proteins, such as IncA interact directly with a subset of host SNARE proteins, specifically VAMPs, to play a proposed role in membrane fusion [72, 73]: IncG interacts with the host protein 14-3-3β [74], which has known roles in several host-cell signaling pathways. Once again the ubiquitous Rab GTPAses are observed on the inclusion membrane, e.g. the early chlamydial Inc protein CT229 interacts with and recruits Rab4 to the inclusion membrane and therefore may play a role in the biogenesis of or trafficking to the inclusion [75]. For comprehensive and insightful reviews of chlamydial effectors involved in the development cycle readers are referred to [76, 77]. As the inclusion expands, the integrity is kept intact via the formation of a stabilizing scaffold around the inclusion comprising F-actin (RhoA dependent recruitment) and intermediate filaments (IF) [78].

(4) Host Cell Exit The infection cycle is complete after 2–3 days when the RBs transform back to the infectious EBs which are then released from the host cell into the external milieu via extrusion or lysis and are then poised to infect new cells or hosts. Actin polymerization, neuronal Wiskott–Aldrich syndrome protein (N-WASP), myosin II and Rho GTPase are required for extrusion, whereas lysis is mediated by proteases (bacterial or cellular) and intracellular calcium [4].

(5) Persistence Persistent infection with *Chlamydia* has been linked to chronic disease sequelae, such as blinding trachoma and pelvic inflammatory disease (PID). Studies *in vitro* have demonstrated that *Chlamydia* starved of essential nutrients e.g. iron [79] enter a state of persistence, representing a viable but non-cultivable growth stage, which allows the establishment of a long-term association with the host. Nutrient deprivation by indoleamine 2,3-dioxygenase (IDO)-mediated tryptophan catabolism from indole and serine (by *TrpRBA*) is thought to be the mechanism by which host cell IFNγ mediates the persistent growth of *C. trachomatis* [80, 81]. During the persistent stage, cell division stops, the RBs become morphologically aberrant by increasing in size and do not mature into

EBs. Recent clinical studies of human and animal disease have also provided some evidence for the existence of chlamydial persistence *in vivo* [82]. In both an *in vitro* model of persistent *C. trachomatis* infection and from individuals with putative *Chlamydia*-induced arthritis, persistence has been related to increased expression of ct604, one of the three heat shock protein (hsp60) coding genes [83].

8.2.2
Genetic Determinants of Infection and Disease

Appraisal of the chlamydial cycle of development as described above, immediately leads to a suggestion of dynamic cross-talk between bacteria and host that ultimately leads to successful infection of host tissues. But why is an infection successful in one host and not another; what are the key determinants within the individual pathogen and host that predispose the infection to a successful outcome?

8.2.2.1 **Chlamydial Factors**

Understanding the *Chlamydia*–host interaction has benefited from the explosion of available genomic information. Since the publication of the *C. trachomatis* serovar D genome in 1998 [84], a further 13 Chlamydiae genomes, including four *C. trachomatis* and four *C. pneumoniae* strains, have been completed and the sequencing of 14 more strains (including 10 *C. trachomatis* and one *C. pneumoniae* strain) is still in progress (See http://www.ncbi.nlm.nih.gov/genomes/lproks.cgi).

Data obtained from these sequencing projects revealed that like many obligate intracellular dwellers, Chlamydiae exhibit a reduced genome size [85], e.g. the *C. trachomatis* genome size is 1.04 Mb [84]. And regardless of ecological niche, *Chlamydia* generally exhibit reductions in the genes providing metabolic diversity, but retain the genes important for pathogenicity, such as those involved in encoding type III secretion systems. Therefore, *Chlamydia* are genetically predisposed to infect various tissue and cell types and evade an ever-adapting host immune system [85, 86].

A limited number of studies have addressed the issue of variation in chlamydial factors and disease susceptibility and severity. Although polymorphisms in the *ompA* gene (encoding MOMP) have been linked to a higher prevalence of infection [87], there is no compelling clinical evidence [88, 89]. Because chlamydial inclusion proteins (Incs) are secreted to the inclusion membrane and exposed to the cytosol, they are regarded as the most likely contenders for direct interactions with host cell cytosol. The *IncA* gene has been implicated in disease severity during infection with *C. trachomatis* [90] but once again this is not supported in a clinical setting [91]. More recently, microarray comparative genome analysis of three major trachoma serotypes was used to show polymorphisms in a small subset of genes, including the translocated actin-recruiting phosphoprotein (TARP) which is correlated with differences in pathogenicity *in vitro* and *in vivo* [92].The authors suggest polymorphisms in the limited number of genes that control pathogenicity of *C. trachomatis* may determine the severity of trachoma. Indeed, Moelleken and co-workers also suggested single position variations in the sequence of chlamydial

genes; in this case the chlamydial outer membrane protein OmcB, required for adhesion to host cells, may reflect differences in cell tropism and disease pattern [40].

A high genetic diversity of *C. trachomatis* has also been linked to the frequency of infection within a community [87]; supporting the notion that diversity is a prerequisite for a high prevalence of infection. A role for a mixed microbial etiology in the severity of disease has also been suggested, e.g. the severity of trachoma sequelae was significantly associated with the viable (i.e. RNA) detection of three *Chlamydia* species [93].

8.2.2.2 **Host Factors**

Since infection with a particular serotype of *Chlamydia* does not always result in the manifestation of clinical symptoms, a host genetic predisposition towards infection is also assumed as likely. Susceptibility and severity of *Chlamydia* infection has been correlated with a number of host genetic factors, mostly of the innate and acquired immune systems, such as human leukocyte antigen (HLA) haplotypes, polymorphisms of cytokine genes and pattern recognition receptors (PRRs) involved in sensing bacterial components, and the expression of chemokines [94–96]. Polymorphisms in the (HLA) class I and II glycoproteins, which play a role in the immune response against infection, have been associated with susceptibility and severity of *Chlamydia* infection in both human subjects and mouse models. In a clinical study of patients with coronary artery disease (CAD), those with the HLA-B*35 allele were at the highest risk of *C. pneumoniae* infection [97]; sex workers with HLA-A31 were found to have an increased risk of pelvic inflammatory disease (PID) [98] and the HLA–A28*6802 allele to significantly increase the risk of *C. trachomatis*-associated trachomatous scarring [99]. Increased severity of scarring was also associated with polymorphisms of the tumor necrosis factor alpha (TNFα) gene [100]. Using genetically modified mice, increased susceptibility to infection resulted from deficiencies in key immune system components such as the cytokine IFN-γ and CD-8 [101]. Most recently, the host chemokine CCL5, which is a potent anti-HIV protein, was identified as important for mounting protective immunity against *Chlamydia* infection in mice [102]. In addition, mice deficient in Myeloid differentiation antigen 88 (MyD88), a key downstream adapter for toll-like receptors (TLRs) and interleukin-1 receptors (IL-1Rs), have increased susceptibility to infections with *C. pneumoniae* [103] (and also other important human intracellular pathogens such as *Mycobacterium tuberculosis* [104, 105] and *Listeria monocytogenes* [106]).

The host factors dictating susceptibility may vary between infection models; for instance, although the primary defense against *Chlamydia* infection in the mouse involves the IFNγ-inducible family of IRG proteins, the mechanisms of host resistance differ substantially between the mouse and human. The IRG protein, Irga6 was identified as an important resistance factor against *C. trachomatis,* but not for infection with the mouse pathogen *C. muridarum* in IFNγ-stimulated mouse embryonic fibroblasts (MEFs) [107]. Similarly, Virok and co-workers [108] showed that the entry-mediated tyrosine phosphorylation of a 70-kDa complex of proteins

was dependent on the strain of *Chlamydia*: infection by human *C. trachomatis* strains, but not the guinea pig pathogen *C. caviae,* induced strong tyrosine phosphorylation of this complex.

The full range of genetic determinants influencing susceptibility to chlamydial infections still remains largely unknown. The list above is dominated by factors associated with the immune system, but other types of host cell determinants that play a role in infection are also likely to be polymorphic. Such polymorphisms are anticipated to modulate susceptibility to and severity of infection. Identification of the key host cell factors would not only impact on the understanding of chlamydial pathogenesis, but may also provide opportunities for the development of improved therapeutics.

8.3
A Global Approach to Investigating the Role of Host Cell Factors during Infection

Owing to the intimate relationship between pathogen and host cell, *Chlamydia* provides an ideal model for investigating the pathogen–host relationship and therefore the ability to link genetic factors with disease susceptibility and severity – an opportunity that is challenged by the genetic intractability of these bacteria. A holistic view of the *Chlamydia*–host interplay has the potential to provide a substantially deeper understanding and allow determination of the key hubs within the complex network of molecular interactions. Capitalizing on the explosion of genomic data in recent years, molecular insight provided by genomic, transcriptomic, proteomics and cutting edge microscopy techniques is now being used to identify interaction hot-spots [109, 110]. Transcriptomic studies on the pathogen side have already revealed the temporal expression of *C. trachomatis* and *C. pneumoniae* genes, whose functional protein products drive the cycle of infection from inclusion formation, acquisition of nutrients, EB to RB differentiation, replication to re-differentiation and persistence [111–114]. Similar analyses have been applied to the host cell side [115–117]. The next step required is the functional validation of these previously implicated or entirely novel factors. But since *Chlamydia* is recalcitrant to genetic manipulation, these functional investigations must be host focused.

8.3.1
RNAi: a New Paradigm to Study Pathogenesis

A recent breakthrough in gene function analysis which overcomes many limitations of both knock-out and RNA anti-sense technologies has been the ability to inhibit the expression of a target host protein via RNA interference (RNAi) (see Figure 8.2). The ability to knock-down a gene (or genes) of interest provides a direct link to its biological functioning and thus provides the possibility of obtaining information of particular patho-physiological relevance and predictive value for the development of new therapeutics.

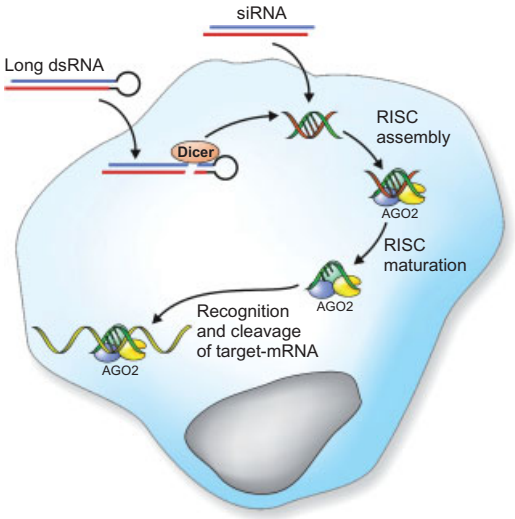


Figure 8.2 The mechanisms of RNA interference (RNAi). Silencing of host cell genes is achieved via RNAi, a naturally-occurring mechanism that plays an important function in eukaryotic gene regulation. Detection of long double-stranded RNA (dsRNA) by the cell elicits the production of an RNase III nuclease, termed DICER, which cleaves long dsRNA into short interfering RNA (siRNA) subunits of 21–23 nucleotides. As the siRNAs unwind they are assembled into a RNA-inducing silencing complex (RISC). Ago2 is thought to be responsible for cleavage of the passenger (sense) strand of the siRNA duplex, leading to activation and maturation of RISC. The antisense RNA strand guides the RISC complex to complementary mRNA, resulting in cleavage and disruption of protein synthesis.

RNAi technology mimics an endogenous cellular defense mechanism against viral attack and replication that results in gene silencing. The elucidation of the mechanisms of RNAi in *C. elegans* [118] led to the development of RNAi technology within insect [119, 120] and mammalian cell lines [121]. The presence of long dsRNA, such as viral RNA, within the cell prompts the production of an RNase III nuclease, termed DICER, which cleaves long dsRNA into short interfering RNA (siRNA) subunits of 21–23 nucleotides. These siRNAs are then incorporated into a RNA-inducing silencing complex (RISC). The RISC proteins vary between species but proteins belonging to the argonaut (Ago) family are consistently found. Ago2 is thought to be responsible for cleavage of the passenger (sense) strand of the siRNA duplex, leading to activation of RISC. The antisense RNA strand and RISC complex then bind to homologous mRNA, resulting in its endonucleolytic cleavage and disruption of protein synthesis [122, 123].

Chemically synthesized siRNAs are delivered directly to target cells or host tissues to provide transient silencing of gene function, or alternatively genetically encoded short hairpin RNA (shRNA) guide strands complementary to sequences within promoter regions are used to down-regulate gene expression through transcriptional silencing. shRNAs are often used in combination with a viral- or

plasmid-based delivery system to provide a more permanent reduction in expression level [124]. The phenotypic/functional outcome of the targeted gene(s) knock down within host cells, e.g. increased/ decreased pathogen replication, innate cellular responses, morphological or physiological consequences, or even changes in antigen presentation, can then be detected on a high throughput basis using microscopy, flow cytometry or chemiluminescence to provide a high content readout [125, 126].

The interpretation of this high-content readout involves a number of stringent quality checks to ensure targets identified are not a result of off-target effects or other biological mechanisms interfering with the phenotype obtained. In this way, the number of false positives and negatives will be reduced. Information about identified hits may be combined with other global techniques and tools, such as microarray analysis, bioinformatics to explore relationships, mechanisms, functions, and pathways of relevance using software such as Ingenuity (http://www.ingenuity.com). Ultimately, functional validation and characterization in knockout gene animal models and by RNAi *in vivo* can be applied. A typical workflow for an RNAi screen for factors affecting *Chlamydia* replication and subsequent hit validation and investigation is shown in Figure 8.3.

The use of RNAi genome-wide analysis of *Drosophila melanogaster* cell lines with its relatively small (14 000 genes) but evolutionary conserved genome size, a lack of redundancy compared to mammals, and a predisposition to double stranded RNA (dsRNA) uptake, makes this insect an attractive genetic host for host cell functional investigations. The main stumbling block to transferring RNAi technology from Drosophila cell lines and tissues to, an arguably more relevant use in, mammalian cells has been the elicitation of a host cell immune response that interferes with the ability of RNAi to reduce gene expression. The delivery of dsRNA, varying in size between 38 and 1662 base pairs [127, 128] was found to elicit an interferon response in mammalian cells [129] that could result in antiviral, growth inhibitory and apoptotic activity in mammalian cells. This obstacle was first overcome by Elbashir and co-workers, who successfully mediated sequence-specific messenger RNA degradation using 21-base pair nucleotide siRNAs with symmetric 2-nucleotide 3′ overhangs to silence mammalian gene expression [121].

The first RNAi screens sought the answers to a wide range of cellular biological questions, such as factors crucial to cell division [130], cell growth and viability [131] and cell signaling [132]. In the context of infection biology, the first high throughput genome-wide RNAi screens (in Drosophila cells) identified high levels of translational machinery as pivotal for successful picornavirus infection of insects and mammals [133]. Since then discovery and insight has evolved to reveal host factors essential for phagocytosis [134–136], bacterial uptake and adhesion [128, 133], and virus replication (see review in [137]) and headway has been made in the search for host factors involved in the entry, replication and survival of many important intracellular pathogens including *Brucella* [138], *Listeria* [139, 140], *Legionella* [141], *Mycobacterium* [135, 142], *Pseudomonas* [136] and *Chlamydia* [47, 143]. Crucially, many factors identified in these studies elicited corresponding phenotypes in mammalian cells. It is of note that recent work (in mammalian cells) has provided insight

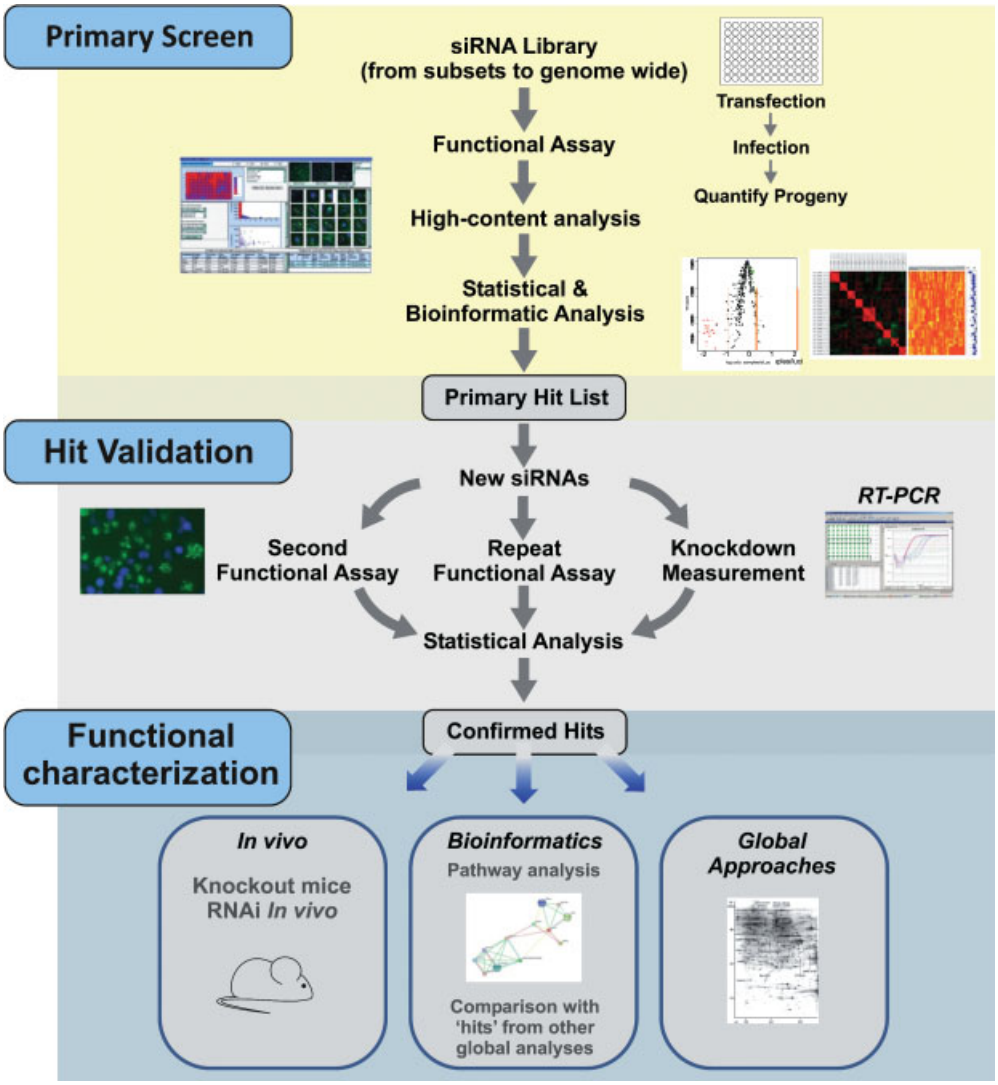


Figure 8.3 From screen to target: Identification and functional validation of host cell factors key to infection. Using a suite of tools ranging from statistics, bioinformatics, plus *in vitro* and *in vivo* robotic experimentation, key host factors identified during RNAi screening are robustly verified and their function is established.

into the human genes affecting West Nile virus host cell entry and infection [144]. Over 21 000 human HeLa cell genes were silenced to demonstrate the critical role of ubiquitin ligase CBLL1 in West Nile virus internalization, a post-entry role for the endoplasmic-reticulum-associated degradation pathway in viral infection, and the monocarboxylic acid transporter MCT4 as important in reducing viral

replication. In addition, the screen confirmed a number of previously identified host factors, confirming the functional capability of this approach.

8.3.2 RNAi and the *Chlamydia*–Host Interaction

The use of Drosophila RNAi screens for *C. trachomatis* has been to a certain extent, limited by the fact that only the early stages of infection occur in these cells [143, 145]. Nevertheless, recent publications have provided genuine insight into the mechanisms of *Chlamydia* host cell entry/uptake. Derre and co-workers used *C. caviae*, an infectious agent of guinea pigs, to investigate the role of host cell factors in *Chlamydia* infection: 31 host cell factors, including the Tom complex, were identified as specific to infection with *C. caviae*; depletion of either Tom40 or Tom22 reduced the burden of infection in mammalian (HeLa) cells [143]. Elwell and co-workers identified the targeting of the TARP actin nucleator by AbI kinase, and PDGFRβ signaling as important, but redundant, host factors in the early stages of infection with *C. trachomatis* [47]. These findings corroborate, in part, previous observations [45, 46]. Interestingly, AbI kinase has also been implicated in the entry/uptake of a number of pathogens including the facultative intracellular bacterium *Pseudomonas aeruginosa* [136].

Numerous studies are now also using RNAi as a complementary technique to aid identification and elucidation of the role of many host factors crucial to the chlamydial development cycle. For instance, Rajalingam and co-workers used siRNAs to down-regulate gene expression and demonstrated a role for inhibitor of apoptosis (IAP-IAP) gene complexes in maintaining apoptosis resistance within *Chlamydia*-infected cells [146] and discovered a critical role for the anti-apoptotic factor Mcl-1 in successful apoptosis resistance [54]. The induction of apoptosis resistance is one of the key mechanisms used by *Chlamydia* to escape host defenses, leading to survival and successful replication within host cells [53, 147]. The mechanics of another novel pathogenesis mechanism were elucidated using siRNA knock-down of Golgi matrix proteins to demonstrate a link between golgin 84 and chlamydial replication, reinforcing the role of Golgi fragmentation in facilitating *C. trachomatis* pathogenesis [66]. The silencing of the host protein ezrin, which serves as a physical link between host cell receptors and the actin cytoskeleton, was found to significantly reduce *C. trachomatis* infectivity of host cells at the stage of entry [148]. Similarly, depletion of WAVE2 and AbI-1 expression in host cells prevented pathogen-induced actin recruitment, thereby significantly reducing the uptake of *C. trachomatis* [45]. Insights into the causal mechanisms of infection-associated disease sequelae, such as coronary heart disease and chronic vascular lesions are also being discovered; for instance, the nucleotide-binding oligomerization domain containing one (*Nod1*) gene was shown to play a role in triggering a *C. pneumoniae*-mediated inflammatory process in epithelial cells [149]. The list of insights gained is steadily increasing as RNAi becomes a more routine part of molecular and cellular investigations.

8.4
What can we Expect from the Global RNAi Approach?

8.4.1
Basic Insights into Cellular Function and Host Susceptibility to Infection

An unprecedented explosion of insight and elucidation can be anticipated from high-throughput global loss-of-function analyses of host–pathogen interactions. While traditional focused investigations in many pathogen models have highlighted many important avenues of cross talk between pathogen and host, we cannot be sure in every case that additional, even more important pathways have not been missed. There are numerous pathogen determinants which supposedly play an essential role in this dialog, however, knowledge of their eukaryotic counterparts, their mechanisms of interaction and their specific roles in the scenario is still lacking. In terms of *Chlamydia*, the novel approach will allow a decoding of the development cycle at every stage, from attachment and entry to exit and persistence induction. For instance, our initial analysis of Golgi matrix components has already revealed that fragmentation of the Golgi apparatus causes an increase in chlamydial replication and could facilitate *Chlamydia* pathogenesis [66]. Thus, the use of RNAi to knock down the function of a cellular factor has provided a direct link to its function.

Having identified and validated an array of crucial host cell factors, e.g. *in vitro* using human cells and in the mouse if it proved to be an adequate *in vivo* model, the diversity of these factors in humans could then be examined. Human gene polymorphisms are in striking abundance and not restricted to typical immune defense genes but exist in almost all gene classes. Conceivably such polymorphisms have a functional impact on both the susceptibility to and severity of infections. An example is given by a recent genome-wide study of the major determinants of HIV virus load: This analysis revealed that nearly 15% of the variability between individuals could be explained by three polymorphisms, emphasizing the importance of host factor variation in determining disease outcome [150]. Very likely, the discovery of novel relationships between individual predisposition and the productive onset and/or course of an infection will be greatly advanced by analyses of systematic host cell function.

8.4.2
A New Therapeutic Concept

The ability of RNAi to accurately silence gene expression is not only providing insight into the mechanisms of pathogenesis and cellular function but also the promise of a novel therapeutic approach[1)] to infectious diseases. In the past, little

1) Other therapeutic approaches are outside the scope of this review. Readers with an interest in this topic should refer to [154–156].

attention has been paid to the theoretical option of blocking host cell rather than pathogen functions. In theory, this option could be substantially advantageous if the substantial variability and mutability of microbial organisms is considered: by targeting essential host cell functions the chances of a microbe developing resistance would be very low, although there would potentially be parallel pathways to deal with, which is often the case in mammalian cells. However, this could be seen as a challenge rather than a knock-out criterion. To date, there have been only a few examples where the course of infection is voluntarily influenced by modulating host cell function. Examples of such cases are the use of proton pump inhibitors in combination with antibiotic eradication of the gastric pathogen *Helicobacter pylori* [151] and the occasional use of steroids prior to antibiotic treatment in cases of bacterial meningitis [152]. In addition, natural remedies traditionally used for the treatment of many infections often target the host rather than the pathogen directly, e.g. curcumin [153] and magnolol [154]. It seems somewhat counter-intuitive to deliberately target host cell determinants against infections: blocking host factors may actually harm the host itself. But it should be taken into account that most medicines we use are designed to cause a (at least transient) block of human gene function. That such treatment is tolerated by the human body is founded in the non-essential character of many human genes, e.g. under conditions of transient or partial blockage. Thus a novel, host factor-oriented approach to treating infections seems to be a realistic notion.

In principle, two strategies could be applied once a relevant 'anti-infective' host cell target has been identified. The first and probably most direct approach is to identify small molecules capable of blocking a host cell function relevant to an infection. This conventional approach, combined with the various facets of, e.g. target structure identification, comprises the standards of contemporary drug screening and development. However, not surprisingly, many gene products that may be potential targets belong to a long list of so-called 'non-druggable targets'. This drawback may be overcome by exploiting the potential of RNAi through one further step: its application to silencing gene expression in the living organism. This novel approach, i.e. the use of RNAi for human therapy (e.g. see RIGHT: www.ip-right.org) promises to extend our current therapeutic repertoire.

Although there is a strong theoretical basis and proof of principle has been established, one or two hurdles still remain before the clinical reality of RNAi as a therapy comes to fruition (for a review see [158]). One of the challenges of RNAi as a therapeutic has been the potential induction of innate immune responses that are directed against double-stranded RNA. However, an innate immune response may be beneficial: generic siRNAs suppressed blinding choroidal neovascularization (an age-related macular degeneration) in mice with comparable efficiency to siRNAs specifically targeting *Vegfa* or *Vegfr1* [159]. Alternatively, efforts are being made to minimize the potential of immune recognition, e.g. by introducing chemical modifications [158, 160]. Moreover, the unintended gene silencing caused by cross-specific mRNA targeting, termed off-target effects, could be reduced by employing algorithms for determining suitable target sequences (e.g., [161]).

Nonetheless, it should be borne in mind that any conventional drug administration also involves more or less pronounced off-target activities. Other major challenges of *in vivo* RNAi include the efficiency and tissue specificity of siRNA delivery; nevertheless, successful local and systemic delivery of modified siRNAs has been reported [162, 163]. Optimization of silencing efficiency, stability, limiting renal excretion and improving targeting in human tissues, represent the current challenges for the use of RNAi as a therapeutic.

Despite the hurdles, in the relatively short time since the discovery of RNAi, reports describing the therapeutic potential of siRNA for human diseases, especially viruses [164] are increasing. Indeed, the development of siRNAs as a treatment for HIV, influenza, hepatitis C and infection with respiratory syncytial virus (ALN-RSV01) has already led to Phase I clinical trials (i.e. using human volunteers) by a variety of pharmaceutical companies [165]. It is foreseeable that similar approaches will be launched to tackle acute as well as chronic bacterial infections, e.g. in combination with conventional antibiotic therapies and vaccination.

8.5 Outlook

An investigation of a topic encompassing 'molecular insight into therapeutic discovery' gives a strong sense that despite a myriad of obstacles, real progress is being made. The advent of RNAi technology as a 'power tool' within the global approach tool-box represents both a new paradigm for investigating host–pathogen interactions and a new therapeutic concept. We now have an approach that can identify key host and bacterial genes essential for inhibiting and/or stimulating the infection process; these factors can then be exploited as novel therapeutic targets and perhaps most intriguingly as a basis for a genetic screen to identify infection-susceptible individuals (see Figure 8.4): knowledge, elucidation and application. The possibilities are clear, but now we must have a modicum of patience as techniques are honed and applications are tested.

Acknowledgments

This work was supported by funding under the Sixth Research Framework Programme of the European Union, Project RIGHT (LSHB-CT-2004-005276) and by the BMBF through the RNAi-Net (Förderkennzeichen 0313938A). We would also like to thank Diane Schad and Dr Nikolaus Machuy for help in producing the figures and Dr Marion Rother for helpful comments on the manuscript.

Apologies to authors within the field not included in this review; due to space limitations the literature cited is not exhaustive.

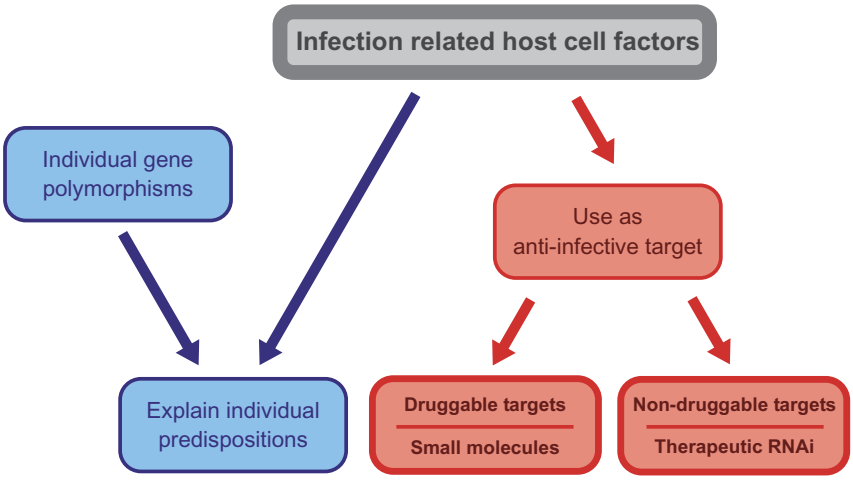


Figure 8.4 Making use of identified infection-related host cell factors. Identifying the key host factors is essential for inhibiting and/or stimulating the infection process and not only reveals novel therapeutic targets, but since human genes are inherently polymorphic, the factors identified could provide a basis for correlating the genetic outfit of individuals with their susceptibility or resistance to infections. Within the therapeutic context, efficient targeting of host cell factors could be achieved by either conventional drug development involving small molecule screening or by directly silencing gene expression via RNAi *in vivo*.

References

1 Gondo, Y. (2008) Trends in large-scale mouse mutagenesis: from genetics to functional genomics. *Nat. Rev. Genet.*, **9**, 803–810.

2 Bhavsar, A.P., Guttman, J.A., and Finlay, B.B. (2007) Manipulation of host-cell pathways by bacterial pathogens. *Nature*, **449**, 827–834.

3 Stevens, J.M., Galyov, E.E., and Stevens, M.P. (2006) Actin-dependent movement of bacterial pathogens. *Nat. Rev. Microbiol.*, **4**, 91–101.

4 Hybiske, K., and Stephens, R.S. (2007) Mechanisms of host cell exit by the intracellular bacterium *Chlamydia*. *Proc. Natl Acad. Sci. USA*, **104**, 11430–11435.

5 Halberstädter, L., and von Prowazek, S. (1907) Zur Ätiologie des Tachoms. *Dtsch. Med. Wochenschr*, **32**, 1285–1287.

6 Carlson, J.H., Porcella, S.F., McClarty, G., and Caldwell, H.D. (2005) Comparative genomic analysis of *Chlamydia trachomatis* oculotropic and genitotropic strains. *Infect. Immun.*, **73**, 6407–6418.

7 Stamm, W.E. (1999) *Chlamydia trachomatis* infections: progress and problems. *J. Infect. Dis.*, **179** (Suppl. 2), S380–S383.

8 Caldwell, H.D., Wood, H., Crane, D., Bailey, R., Jones, R.B., Mabey, D., Maclean, I., Mohammed, Z., Peeling, R., Roshick, C., Schachter, J., Solomon, A.W., Stamm, W.E., Suchland, R.J., Taylor, L., West, S.K., Quinn, T.C., Belland, R.J., and McClarty, G. (2003) Polymorphisms in *Chlamydia trachomatis* tryptophan synthase genes differentiate between genital and ocular isolates. *J. Clin. Invest.*, **111**, 1757–1769.

9 Koh, W.P., Taylor, M.B., Chew, S.K., Phoon, M.C., Kang, K.L., and Chow, V.T. (2003) *Chlamydia pneumoniae* IgG seropositivity and clinical history of ischemic heart disease in Singapore.

J. Microbiol. Immunol. Infect., **36**, 169–174.

10 Stallings, T.L. (2008) Association of Alzheimer's disease and *Chlamydophila pneumoniae*. *J. Infect.*, **56**, 423–431.

11 Saikku, P., Leinonen, M., Mattila, K., Ekman, M.R., Nieminen, M.S., Makela, P.H., Huttunen, J.K., and Valtonen, V. (1988) Serological evidence of an association of a novel Chlamydia, TWAR, with chronic coronary heart disease and acute myocardial infarction. *Lancet*, **2**, 983–986.

12 Beutler, A.M., Whittum-Hudson, J.A., Nanagara, R., Schumacher, H.R., and Hudson, A.P. (1994) Intracellular location of inapparently infecting *Chlamydia* in synovial tissue from patients with Reiter's syndrome. *Immunol. Res.*, **13**, 163–171.

13 von Hertzen, L., Isoaho, R., Leinonen, M., Koskinen, R., Laippala, P., Toyryla, M., Kivela, S.L., and Saikku, P. (1996) *Chlamydia pneumoniae* antibodies in chronic obstructive pulmonary disease. *Int. J. Epidemiol.*, **25**, 658–664.

14 Horn, M. (2008) Chlamydiae as symbionts in eukaryotes. *Annu. Rev. Microbiol.*, **62**, 113–131.

15 Corsaro, D., Feroldi, V., Saucedo, G., Ribas, F., Loret, J.F., and Greub, G. (2009) Novel Chlamydiales strains isolated from a water treatment plant. *Environ. Microbiol.*, **11**, 188–200.

16 Everett, K.D., Bush, R.M., and Andersen, A.A. (1999) Emended description of the order Chlamydiales, proposal of Parachlamydiaceae fam. nov. and Simkaniaceae fam. nov., each containing one monotypic genus, revised taxonomy of the family Chlamydiaceae, including a new genus and five new species, and standards for the identification of organisms. *Int. J. Syst. Bacteriol.*, **49** (Pt 2), 415–440.

17 Kahane, S., Greenberg, D., Friedman, M.G., Haikin, H., and Dagan, R. (1998) High prevalence of 'Simkania Z,' a novel Chlamydia-like bacterium, in infants with acute bronchiolitis. *J. Infect. Dis.*, **177**, 1425–1429.

18 Lieberman, D., Kahane, S., Lieberman, D., and Friedman, M.G. (1997) Pneumonia with serological evidence of acute infection with the *Chlamydia*-like microorganism 'Z'. *Am. J. Respir. Crit. Care Med.*, **156**, 578–582.

19 Fasoli, L., Paldanius, M., Don, M., Valent, F., Vetrugno, L., Korppi, M., and Canciani, M. (2008) *Simkania negevensis* in community-acquired pneumonia in Italian children. *Scand. J. Infect. Dis.*, **40**, 269–272.

20 Harkinezhad, T., Geens, T., and Vanrompay, D. (2008) *Chlamydophila psittaci* infections in birds: a review with emphasis on zoonotic consequences. *Vet. Microbiol.*. doi: 10.1016/j.vetmic.2008.09.047

21 Read, T.D., Brunham, R.C., Shen, C., Gill, S.R., Heidelberg, J.F., White, O., Hickey, E.K., Peterson, J., Utterback, T., Berry, K., Bass, S., Linher, K., Weidman, J., Khouri, H., Craven, B., Bowman, C., Dodson, R., Gwinn, M., Nelson, W., DeBoy, R., Kolonay, J., McClarty, G., Salzberg, S.L., Eisen, J., and Fraser, C.M. (2000) Genome sequences of *Chlamydia trachomatis* MoPn and *Chlamydia pneumoniae* AR39. *Nucleic Acids Res.*, **28**, 1397–1406.

22 Lefevre, J.C., Lepargneur, J.P., Guion, D., and Bei, S. (1997) Tetracycline-resistant *Chlamydia trachomatis* in Toulouse, France. *Pathol. Biol. (Paris) Sex. Transm. Dis.*, **45**, 376–378.

23 Mourad, A., Sweet, R.L., Sugg, N., and Schachter, J. (1980) Relative resistance to erythromycin in *Chlamydia trachomatis*. *Antimicrob. Agents Chemother.*, **18**, 696–698.

24 Samra, Z., Rosenberg, S., Soffer, Y., and Dan, M. (2001) *In vitro* susceptibility of recent clinical isolates of Chlamydia trachomatis to macrolides and tetracyclines. *Diagn. Microbiol. Infect. Dis.*, **39**, 177–179.

25 Somani, J., Bhullar, V.B., Workowski, K.A., Farshy, C.E., and Black, C.M. (2000) Multiple drug-resistant *Chlamydia trachomatis* associated with clinical treatment failure. *J. Infect. Dis.*, **181**, 1421–1427.

26 Yamaguchi, H., Friedman, H., Yamamoto, M., Yasuda, K., and Yamamoto, Y. (2003) *Chlamydia pneumoniae* resists antibiotics in

lymphocytes. *Antimicrob. Agents Chemother.*, **47**, 1972–1975.

27 Beatty, W.L., Byrne, G.I., and Morrison, R.P. (1993) Morphologic and antigenic characterization of interferon gamma-mediated persistent *Chlamydia trachomatis* infection *in vitro*. *Proc. Natl Acad. Sci. USA*, **90**, 3998–4002.

28 Beatty, W.L., Morrison, R.P., and Byrne, G.I. (1994) Persistent chlamydiae: from cell culture to a paradigm for chlamydial pathogenesis. *Microbiol. Rev.*, **58**, 686–699.

29 Mpiga, P., and Ravaoarinoro, M. (2006) Effects of sustained antibiotic bactericidal treatment on *Chlamydia trachomatis*-infected epithelial-like cells (HeLa) and monocyte-like cells (THP-1 and U-937). *Int. J. Antimicrob. Agents*, **27**, 316–324.

30 Projan, S.J., and Bradford, P.A. (2007) Late stage antibacterial drugs in the clinical pipeline. *Curr. Opin. Microbiol.*, **10**, 441–446.

31 Becker, D., Selbach, M., Rollenhagen, C., Ballmaier, M., Meyer, T.F., Mann, M., and Bumann, D. (2006) Robust *Salmonella* metabolism limits possibilities for new antimicrobials. *Nature*, **440**, 303–307.

32 Barker, C.J., Beagley, K.W., Hafner, L.M., and Timms, P. (2008) In silico identification and *in vivo* analysis of a novel T-cell antigen from *Chlamydia*, NrdB. *Vaccine*, **26**, 1285–1296.

33 Hansen, J., Jensen, K.T., Follmann, F., Agger, E.M., Theisen, M., and Andersen, P. (2008) Liposome delivery of *Chlamydia muridarum* major outer membrane protein primes a Th1 response that protects against genital chlamydial infection in a mouse model. *J. Infect. Dis.*, **198**, 758–767.

34 Li, W., Murthy, A.K., Guentzel, M.N., Seshu, J., Forsthuber, T.G., Zhong, G., and Arulanandam, B.P. (2008) Antigen-specific CD4+ T cells produce sufficient IFN-gamma to mediate robust protective immunity against genital *Chlamydia muridarum* infection. *J. Immunol.*, **180**, 3375–3382.

35 Hafner, L.M., and McNeilly, C. (2008) Vaccines for *Chlamydia* infections of the female genital tract. *Future Microbiol.*, **3**, 67–77.

36 Clifton, D.R., Fields, K.A., Grieshaber, S.S., Dooley, C.A., Fischer, E.R., Mead, D.J., Carabeo, R.A., and Hackstadt, T. (2004) A chlamydial type III translocated protein is tyrosine-phosphorylated at the site of entry and associated with recruitment of actin. *Proc. Natl Acad. Sci. USA*, **101**, 10166–10171.

37 Stephens, R.S., Poteralski, J.M., and Olinger, L. (2006) *Interaction* of *Chlamydia trachomatis* with mammalian cells is independent of host cell surface heparan sulfate glycosaminoglycans. *Infect. Immun.*, **74**, 1795–1799.

38 Zhang, J.P., and Stephens, R.S. (1992) Mechanism of *C. trachomatis* attachment to eukaryotic host cells. *Cell*, **69**, 861–869.

39 Fadel, S., and Eley, A. (2007) *Chlamydia trachomatis* OmcB protein is a surface-exposed glycosaminoglycan-dependent adhesin. *J. Med. Microbiol.*, **56**, 15–22.

40 Moelleken, K., and Hegemann, J.H. (2008) The *Chlamydia* outer membrane protein OmcB is required for adhesion and exhibits biovar-specific differences in glycosaminoglycan binding. *Mol. Microbiol.*, **67**, 403–419.

41 Su, H., Watkins, N.G., Zhang, Y.X., and Caldwell, H.D. (1990) *Chlamydia trachomatis*-host cell interactions: role of the chlamydial major outer membrane protein as an adhesin. *Infect. Immun.*, **58**, 1017–1025.

42 Conant, C.G., and Stephens, R.S. (2007) *Chlamydia* attachment to mammalian cells requires protein disulfide isomerase. *Cell Microbiol.*, **9**, 222–232.

43 Carabeo, R.A., Grieshaber, S.S., Fischer, E., and Hackstadt, T. (2002) *Chlamydia trachomatis* induces remodeling of the actin cytoskeleton during attachment and entry into HeLa cells. *Infect. Immun.*, **70**, 3793–3803.

44 Carabeo, R.A., Grieshaber, S.S., Hasenkrug, A., Dooley, C., and Hackstadt, T. (2004) Requirement for the Rac GTPase in *Chlamydia trachomatis* invasion of non-phagocytic cells. *Traffic*, **5**, 418–425.

45 Carabeo, R.A., Dooley, C.A., Grieshaber, S.S., and Hackstadt, T. (2007) Rac interacts with Abi-1 and WAVE2 to promote an Arp2/3-dependent actin recruitment during chlamydial invasion. *Cell Microbiol.*, **9**, 2278–2288.

46 Jewett, T.J., Fischer, E.R., Mead, D.J., and Hackstadt, T. (2006) Chlamydial TARP is a bacterial nucleator of actin. *Proc. Natl Acad. Sci. USA*, **103**, 15599–15604.

47 Elwell, C.A., Ceesay A., Kim J.H., Kalman D., and Engel J.N. (2008) RNA Interference screen identifies Abl Kinase and PDGFR signaling in *Chlamydia trachomatis* entry. *PloS Pathog.*, **4**, 1–15.

48 Mehlitz, A., Banhart, S., Hess, S., Selbach, M., and Meyer, T. (2008) Complex kinase requirements for *Chlamydia trachomatis* Tarp phosphorylation. *FEMS Microbiol. Lett.*, **289**, 233–240.

49 Fields, K.A., and Hackstadt, T. (2002) The chlamydial inclusion: escape from the endocytic pathway. *Annu. Rev. Cell Dev. Biol.*, **18**, 221–245.

50 Heinzen, R.A., Scidmore, M.A., Rockey, D.D., and Hackstadt, T. (1996) Differential interaction with endocytic and exocytic pathways distinguish parasitophorous vacuoles of *Coxiella burnetii* and *Chlamydia trachomatis*. *Infect. Immun.*, **64**, 796–809.

51 Scidmore, M.A., Fischer, E.R., and Hackstadt, T. (2003) Restricted fusion of *Chlamydia trachomatis* vesicles with endocytic compartments during the initial stages of infection. *Infect. Immun.*, **71**, 973–984.

52 van Ooij, C., Apodaca, G., and Engel, J. (1997) Characterization of the *Chlamydia trachomatis* vacuole and its interaction with the host endocytic pathway in HeLa cells. *Infect. Immun.*, **65**, 758–766.

53 Fan, T., Lu, H., Hu, H., Shi, L., McClarty, G.A., Nance, D.M., Greenberg, A.H., and Zhong, G. (1998) Inhibition of apoptosis in chlamydia-infected cells: blockade of mitochondrial cytochrome c release and caspase activation. *J. Exp. Med.*, **187**, 487–496.

54 Rajalingam, K., Sharma, M., Lohmann, C., Oswald, M., Thieck, O., Froelich, C.J., and Rudel, T. (2008) Mcl-1 is a key regulator of apoptosis resistance in *Chlamydia trachomatis*-infected cells. *PLoS ONE*, **3**, e3102.

55 Belland, R.J., Zhong, G., Crane, D.D., Hogan, D., Sturdevant, D., Sharma, J., Beatty, W.L., and Caldwell, H.D. (2003) Genomic transcriptional profiling of the developmental cycle of *Chlamydia trachomatis*. *Proc. Natl Acad. Sci. USA*, **100**, 8478–8483.

56 Clausen, J.D., Christiansen, G., Holst, H.U., and Birkelund, S. (1997) *Chlamydia trachomatis* utilizes the host cell microtubule network during early events of infection. *Mol. Microbiol.*, **25**, 441–449.

57 Grieshaber, S.S., Grieshaber, N.A., and Hackstadt, T. (2003) *Chlamydia trachomatis* uses host cell dynein to traffic to the microtubule-organizing center in a p50 dynamitin-independent process. *J. Cell Sci.*, **116**, 3793–3802.

58 Tuvim, M.J., Adachi, R., Hoffenberg, S., and Dickey, B.F. (2001) Traffic control: Rab GTPases and the regulation of interorganellar transport. *News Physiol. Sci.*, **16**, 56–61.

59 Carabeo, R.A., Mead, D.J., and Hackstadt, T. (2003) Golgi-dependent transport of cholesterol to the *Chlamydia trachomatis* inclusion. *Proc. Natl Acad. Sci. USA*, **100**, 6771–6776.

60 Hackstadt, T., Scidmore, M.A., and Rockey, D.D. (1995) Lipid metabolism in *Chlamydia trachomatis*-infected cells: directed trafficking of Golgi-derived sphingolipids to the chlamydial inclusion. *Proc. Natl Acad. Sci. USA*, **92**, 4877–4881.

61 Hackstadt, T., Rockey, D.D., Heinzen, R.A., and Scidmore, M.A. (1996) *Chlamydia trachomatis* interrupts an exocytic pathway to acquire endogenously synthesized sphingomyelin in transit from the Golgi apparatus to the plasma membrane. *EMBO J.*, **15**, 964–977.

62 Beatty, W.L. (2006) Trafficking from CD63-positive late endocytic multivesicular bodies is essential for

intracellular development of *Chlamydia trachomatis*. *J. Cell Sci.*, **119**, 350–359.

63 Cocchiaro, J.L., Kumar, Y., Fischer, E.R., Hackstadt, T., and Valdivia, R.H. (2008) Cytoplasmic lipid droplets are translocated into the lumen of the *Chlamydia trachomatis* parasitophorous vacuole. *Proc. Natl Acad. Sci. USA*, **105**, 9379–9384.

64 Rockey, D.D., Viratyosin, W., Bannantine, J.P., Suchland, R.J., and Stamm, W.E. (2002) Diversity within inc genes of clinical *Chlamydia trachomatis* variant isolates that occupy non-fusogenic inclusions. *Microbiology*, **148**, 2497–2505.

65 Su, H., McClarty, G., Dong, F., Hatch, G.M., Pan, Z.K., and Zhong, G. (2004) Activation of Raf/MEK/ERK/cPLA2 signaling pathway is essential for chlamydial acquisition of host glycerophospholipids. *J. Biol. Chem.*, **279**, 9409–9416.

66 Heuer, D., Reyman Lipinski, A., Machuy, N., Karlas, A., Wehrens, A., Siedler, F., Brinkmann, V., and Meyer, T. F. (2009) *Chlamydia* causes fragmentation of the Golgi compartment to ensure reproduction. *Nature*, **457**, 731–735.

67 Fischer, S.F., Vier, J., Kirschnek, S., Klos, A., Hess, S., Ying, S., and Hacker, G. (2004) *Chlamydia* inhibit host cell apoptosis by degradation of proapoptotic BH3-only proteins. *J. Exp. Med.*, **200**, 905–916.

68 Le Negrate, G., Krieg, A., Faustin, B., Loeffler, M., Godzik, A., Krajewski, S., and Reed, J.C. (2008) ChlaDub1 of *Chlamydia trachomatis* suppresses NF-kappaB activation and inhibits IkappaBalpha ubiquitination and degradation. *Cell Microbiol.*, **10**, 1879–1892.

69 Lad, S.P., Li, J., da Silva, C.J., Pan, Q., Gadwal, S., Ulevitch, R.J., and Li, E. (2007) Cleavage of p65/RelA of the NF-kappaB pathway by *Chlamydia*. *Proc. Natl Acad. Sci. USA*, **104**, 2933–2938.

70 Zhong, G., Liu, L., Fan, T., Fan, P., and Ji, H. (2000) Degradation of transcription factor RFX5 during the inhibition of both constitutive and interferon gamma-inducible major histocompatibility complex class I expression in Chlamydia-infected cells. *J. Exp. Med.*, **191**, 1525–1534.

71 Heuer, D., Brinkmann, V., Meyer, T.F., and Szczepek, A.J. (2003) Expression and translocation of chlamydial protease during acute and persistent infection of the epithelial HEp-2 cells with *Chlamydophila (Chlamydia) pneumoniae*. *Cell Microbiol.*, **5**, 315–322.

72 Delevoye, C., Nilges, M., Dautry-Varsat, A., and Subtil, A. (2004) Conservation of the biochemical properties of IncA from *Chlamydia trachomatis* and *Chlamydia caviae*: oligomerization of IncA mediates interaction between facing membranes. *J. Biol. Chem.*, **279**, 46896–46906.

73 Delevoye, C., Nilges, M., Dehoux, P., Paumet, F., Perrinet, S., Dautry-Varsat, A., and Subtil, A. (2008) SNARE protein mimicry by an intracellular bacterium. *PLoS Pathog.*, **4**, e1000022.

74 Scidmore, M.A., and Hackstadt, T. (2001) Mammalian 14-3-3beta associates with the *Chlamydia trachomatis* inclusion membrane via its interaction with IncG. *Mol. Microbiol.*, **39**, 1638–1650.

75 Rzomp, K.A., Scholtes, L.D., Briggs, B.J., Whittaker, G.R., and Scidmore, M.A. (2003) Rab GTPases are recruited to chlamydial inclusions in both a species-dependent and species-independent manner. *Infect. Immun.*, **71**, 5855–5870.

76 Brumell, J.H., Scidmore, M.A. (2007) Manipulation of Rab GTPase function by intracellular bacterial pathogens. *Microbiol. Mol. Biol. Rev.*, **71**, 636–652.

77 Valdivia, R.H. (2008) *Chlamydia* effector proteins and new insights into chlamydial cellular microbiology. *Curr. Opin. Microbiol.*, **11**, 53–59.

78 Kumar, Y., and Valdivia, R.H. (2008) Actin and intermediate filaments stabilize the *Chlamydia trachomatis* vacuole by forming dynamic structural scaffolds. *Cell Host Microbe*, **4**, 159–169.

79 Al Younes, H.M., Rudel, T., Brinkmann, V., Szczepek, A.J., and Meyer, T.F. (2001) Low iron availability modulates the course of *Chlamydia pneumoniae* infection. *Cell Microbiol.*, **3**, 427–437.

80 Beatty, W.L., Belanger, T.A., Desai, A.A., Morrison, R.P., and Byrne, G.I. (1994) Tryptophan depletion as a mechanism of gamma interferon-mediated chlamydial persistence. *Infect. Immun.*, **62**, 3705–3711.

81 Leonhardt, R.M., Lee, S.J., Kavathas, P.B., and Cresswell, P. (2007) Severe tryptophan starvation blocks onset of conventional persistence and reduces reactivation of *Chlamydia trachomatis*. *Infect. Immun.*, **75**, 5105–5117.

82 Hogan, R.J., Mathews, S.A., Mukhopadhyay, S., Summersgill, J.T., and Timms, P. (2004) Chlamydial persistence: beyond the biphasic paradigm. *Infect. Immun.*, **72**, 1843–1855.

83 Gérard, H.C., Whittum-Hudson, J.A., Schumacher, Jr.R., and Hudson, A.P. (2008) Differential expression of three *Chlamydia trachomatis* hsp60-encoding genes in active vs. persistent infections. *Microb. Pathog.*, **36**, 35–49.

84 Stephens, R.S., Kalman, S., Lammel, C., Fan, J., Marathe, R., Aravind, L., Mitchell, W., Olinger, L., Tatusov, R.L., Zhao, Q., Koonin, E.V., and Davis, R.W. (1998) Genome sequence of an obligate intracellular pathogen of humans: *Chlamydia trachomatis*. *Science*, **282**, 754–759.

85 Wernegreen, J.J. (2005) For better or worse: genomic consequences of intracellular mutualism and parasitism. *Curr. Opin. Genet. Dev.*, **15**, 572–583.

86 Subtil, A., and Dautry-Varsat, A. (2004) *Chlamydia*: five years A.G. (after genome) *Curr. Opin. Microbiol.*, **7**, 85–92.

87 Zhang, J., Lietman, T., Olinger, L., Miao, Y., and Stephens, R.S. (2004) Genetic diversity of *Chlamydia trachomatis* and the prevalence of trachoma. *Pediatr. Infect. Dis. J.*, **23**, 217–220.

88 Bailey, R.L., Hayes, L., Pickett, M., Whittle, H.C., Ward, M.E., and Mabey, D.C. (1994) Molecular epidemiology of trachoma in a Gambian village. *Br. J. Ophthalmol.*, **78**, 813–817.

89 Millman, K., Black, C.M., Stamm, W.E., Jones, R.B., Hook, E.W., III, Martin, D.H., Bolan, G., Tavare, S., and Dean, D. (2006) Population-based genetic epidemiologic analysis of *Chlamydia trachomatis* serotypes and lack of association between ompA polymorphisms and clinical phenotypes. *Microbes. Infect.*, **8**, 604–611.

90 Suchland, R.J., Jeffrey, B.M., Xia, M., Bhatia, A., Chu, H.G., Rockey, D.D., and Stamm, W.E. (2008) Identification of concomitant infection with *Chlamydia trachomatis* IncA-negative mutant and wild-type strains by genomic, transcriptional, and biological characterizations. *Infect. Immun.*, **76**, 5438–5446.

91 Pannekoek, Y., Spaargaren, J., Langerak, A.A., Merks, J., Morre, S.A., and van der, Ende, A. (2005) Interrelationship between polymorphisms of incA, fusogenic properties of *Chlamydia trachomatis* strains, and clinical manifestations in patients in The Netherlands. *J. Clin. Microbiol.*, **43**, 2441–2443.

92 Kari, L., Whitmire, W.M., Carlson, J.H., Crane, D.D., Reveneau, N., Nelson, D.E., Mabey, D.C., Bailey, R.L., Holland, M.J., McClarty, G., and Caldwell, H.D. (2008) Pathogenic diversity among *Chlamydia trachomatis* ocular strains in nonhuman primates is affected by subtle genomic variations. *J. Infect. Dis.*, **197**, 449–456.

93 Dean, D., Kandel, R.P., Adhikari, H.K., and Hessel, T. (2008) Multiple Chlamydiaceae species in trachoma: implications for disease pathogenesis and control. *PLoS Med.*, **5**, e14.

94 den Hartog, J.E., Ouburg, S., Land, J.A., Lyons, J.M., Ito, J.I., Pena, A.S., and Morre, S.A. (2006) Do host genetic traits in the bacterial sensing system play a role in the development of *Chlamydia trachomatis*-associated tubal pathology in subfertile women? *BMC Infect. Dis.*, **6**, 122.

95 Rupp, J., Goepel, W., Kramme, E., Jahn, J., Solbach, W., and Maass, M. (2004) CD14 promoter polymorphism -159C>T is associated with susceptibility to chronic *Chlamydia pneumoniae* infection in peripheral blood monocytes. *Genes Immun.*, **5**, 435–438.

96 Wang, C., Tang, J., Geisler, W.M., Crowley-Nowick, P.A., Wilson, C.M., and Kaslow, R.A. (2005) Human

leukocyte antigen and cytokine gene variants as predictors of recurrent *Chlamydia trachomatis* infection in high-risk adolescents. *J. Infect. Dis.*, **191**, 1084–1092.

97 Palikhe, A., Lokki, M.-L., Saikku, P., Leinonen, M., Paldanius, M., Seppänen, M., Valtonen, V., Nieminen, M.-S., and Sinisalo, J. (2008) Association of *Chlamydia pneumoniae* infection with HLA-B*35 in patients with coronary artery disease. *Clin. Vaccine Immunol.*, **15**, 55–59.

98 Kimani, J., Maclean, I.W., Bwayo, J.J., MacDonald, K., Oyugi, J., Maitha, G.M., Peeling, R.W., Cheang, M., Nagelkerke, N.J., Plummer, F.A., and Brunham, R.C. (1996) Risk factors for *Chlamydia trachomatis* pelvic inflammatory disease among sex workers in Nairobi, Kenya. *J. Infect. Dis.*, **173**, 1437–1444.

99 Conway, D.J., Holland, M.J., Campbell, A.E., Bailey, R.L., Krausa, P., Peeling, R.W., Whittle, H.C., and Mabey, D.C. (1996) HLA class I and II polymorphisms and trachomatous scarring in a *Chlamydia trachomatis*-endemic population. *J. Infect. Dis.*, **174**, 643–646.

100 Conway, D.J., Holland, M.J., Bailey, R.L., Campbell, A.E., Mahdi, O.S., Jennings, R., Mbena, E., and Mabey, D.C. (1997) Scarring trachoma is associated with polymorphism in the tumor necrosis factor alpha (TNF-alpha) gene promoter and with elevated TNF-alpha levels in tear fluid. *Infect. Immun.*, **65**, 1003–1006.

101 Rottenberg, M.E., Gigliotti-Rothfuchs, A., and Wigzell, H. (2002) The role of IFN-gamma in the outcome of chlamydial infection. *Curr. Opin. Immunol.*, **14**, 444–451.

102 Sakthivel, S.K., Singh, U.P., Singh, S., Taub, D.D., Igietseme, J.U., and Lillard, J.W., Jr. (2008) CCL5 regulation of mucosal chlamydial immunity and infection. *BMC Microbiol.*, **8**, 136.

103 Naiki, Y., Michelsen, K.S., Schroder, N.W., Alsabeh, R., Slepenkin, A., Zhang, W., Chen, S., Wei, B., Bulut, Y., Wong, M.H., Peterson, E.M., and Arditi, M. (2005) MyD88 is pivotal for the early inflammatory response and subsequent bacterial clearance and survival in a mouse model of *Chlamydia pneumonia* pneumonia. *J. Biol. Chem.*, **280**, 29242–29249.

104 Scanga, C.A., Bafica, A., Feng, C.G., Cheever, A.W., Hieny, S., and Sher, A. (2004) MyD88-deficient mice display a profound loss in resistance to *Mycobacterium tuberculosis* associated with partially impaired Th1 cytokine and nitric oxide synthase 2 expression. *Infect. Immun.*, **72**, 2400–2404.

105 Sugawara, I., Yamada, H., Mizuno, S., Takeda, K., and Akira, S. (2003) Mycobacterial infection in MyD88-deficient mice. *Microbiol. Immunol.*, **47**, 841–847.

106 Torres, D., Barrier, M., Bihl, F., Quesniaux, V.J., Maillet, I., Akira, S., Ryffel, B., and Erard, F. (2004) Toll-like receptor 2 is required for optimal control of *Listeria monocytogenes* infection. *Infect. Immun.*, **72**, 2131–2139.

107 Al-Zeer, M., Al-Younes, HM., Braun, P., Zerrhan, J., and Meyer, T. (2009) IFN-gamma-inducible Irga6 mediates host resistance against *Chlamydia trachomatis* via autophagy. *PLoS ONE*, **4**, e4588.

108 Virok, D.P., Nelson, D.E., Whitmire, W.M., Crane, D.D., Goheen, M.M., and Caldwell, H.D. (2005) Chlamydial infection induces pathobiotype-specific protein tyrosine phosphorylation in epithelial cells. *Infect. Immun.*, **73**, 1939–1946.

109 Jenner, R.G., and Young, R.A. (2005) Insights into host responses against pathogens from transcriptional profiling. *Nat. Rev. Microbiol.*, **3**, 281–294.

110 Walduck, A., Rudel, T., and Meyer, T.F. (2004) Proteomic and gene profiling approaches to study host responses to bacterial infection. *Curr. Opin. Microbiol.*, **7**, 33–38.

111 Abdelrahman, Y.M., and Belland, R.J. (2005) The chlamydial developmental cycle. *FEMS Microbiol. Rev.*, **29**, 949–959.

112 Dill, B.D., Dessus-Babus, S., and Raulston, J.E. (2009) Identification of iron-responsive proteins expressed by *Chlamydia trachomatis* reticulate bodies during intracellular growth. *Microbiology*, **155**, 210–219.

113 Maurer, A.P., Mehlitz, A., Mollenkopf, H.J., and Meyer, T.F. (2007) Gene expression profiles of *Chlamydophila pneumoniae* during the developmental cycle and iron depletion-mediated persistence. *PLoS Pathog.*, **3**, e83.

114 Shaw, E.I., Dooley, C.A., Fischer, E.R., Scidmore, M.A., Fields, K.A., and Hackstadt, T. (2000) Three temporal classes of gene expression during the *Chlamydia trachomatis* developmental cycle. *Mol. Microbiol.*, **37**, 913–925.

115 Mahony, J.B. (2002) Chlamydiae host cell interactions revealed using DNA microarrays. *Ann. NY Acad. Sci.*, **975**, 192–201.

116 Nelson, D.E., Virok, D.P., Wood, H., Roshick, C., Johnson, R.M., Whitmire, W.M., Crane, D.D., Steele-Mortimer, O., Kari, L., McClarty, G., and Caldwell, H.D. (2005) Chlamydial IFN-gamma immune evasion is linked to host infection tropism. *Proc. Natl Acad. Sci. USA*, **102**, 10658–10663.

117 Schrader, S., Klos, A., Hess, S., Zeidler, H., Kuipers, J.G., and Rihl, M. (2007) Expression of inflammatory host genes in *Chlamydia trachomatis*-infected human monocytes. *Arthritis Res. Ther.*, **9**, R54.

118 Fire, A., Xu, S., Hontgomery, M.K., Kostas, S.A., Driver, S.E., and Mello, C.C. (1998) Specific genetic interference by double-stranded RNA in *Caenorhabditis elegans*. *Nature*, **391**, 806–811.

119 Hammond, S.M., Bernstein, E., Beach, D., and Hannon, G.J. (2000) An RNA-directed nuclease mediates post-transcriptional gene silencing in Drosophila cells. *Nature*, **404**, 293–296.

120 Kennerdell, J.R., and Carthew, R.W. (1998) Use of dsRNA-mediated genetic interference to demonstrate that frizzled and frizzled 2 act in the wingless pathway. *Cell*, **95**, 1017–1026.

121 Elbashir, S.M., Harborth, J., Lendeckel, W., Yalcin, A., Weber, K., and Tuschl, T. (2001) Duplexes of 21-nucleotide RNAs mediate RNA interference in cultured mammalian cells. *Nature*, **411**, 494–498.

122 McManus, M.T., and Sharp, P.A. (2002) Gene silencing in mammals by small interfering RNAs. *Nat. Rev. Genet.*, **3**, 737–747.

123 Megha Ghildiyal, M., and Zamore, P.D. (2009) Small silencing RNAs: an expanding universe. *Nat. Rev. Gen.*, **10**, 94–108.

124 Paddison, P.J., Caudy, A.A., Bernstein, E., Hannon, G.J., and Conklin, D.S. (2002) Short hairpin RNAs (shRNAs) induce sequence-specific silencing in mammalian cells. *Genes Dev.*, **16**, 948–958.

125 Echeverri, C.J., and Perrimon, N. (2006) High-throughput RNAi screening in cultured cells: a user's guide. *Nat. Rev. Genet.*, **7**, 373–384.

126 Neumann, B., Held, M., Liebel, U., Erfle, H., Rogers, P., Pepperkok, R., and Ellenberg, J. (2006) High-throughput RNAi screening by time-lapse imaging of live human cells. *Nat. Methods*, **3**, 385–390.

127 Caplen, N.J., Fleenor, J., Fire, A., and Morgan, R.A. (2000) dsRNA-mediated gene silencing in cultured Drosophila cells: a tissue culture model for the analysis of RNA interference. *Gene*, **252**, 95–105.

128 Ui-Tei, K., Zenno, S., Miyata, Y., and Saigo, K. (2000) Sensitive assay of RNA interference in Drosophila and Chinese hamster cultured cells using firefly luciferase gene as target. *FEBS Lett.*, **479**, 79–82.

129 Stark, G.R., Kerr, I.M., Williams, B.R., Silverman, R.H., and Schreiber, R.D. (1998) How cells respond to interferons. *Annu. Rev. Biochem.*, **67**, 227–264.

130 Gonczy, P., Echeverri, C., Oegema, K., Coulson, A., Jones, S.J., Copley, R.R., Duperon, J., Oegema, J., Brehm, M., Cassin, E., Hannak, E., Kirkham, M., Pichler, S., Flohrs, K., Goessen, A., Leidel, S., Alleaume, A.M., Martin, C., Ozlu, N., Bork, P., and Hyman, A.A.

(2000) Functional genomic analysis of cell division in *C. elegans* using RNAi of genes on chromosome III. *Nature*, **408**, 331–336.

131 Boutros, M., Kiger, A.A., Armknecht, S., Kerr, K., Hild, M., Koch, B., Haas, S.A., Paro, R., and Perrimon, N. (2004) Genome-wide RNAi analysis of growth and viability in Drosophila cells. *Science*, **303**, 832–835.

132 Friedman, A., and Perrimon, N. (2006) A functional RNAi screen for regulators of receptor tyrosine kinase and ERK signalling. *Nature*, **444**, 230–234.

133 Cherry, S., Doukas, T., Armknecht, S., Whelan, S., Wang, H., Sarnow, P., and Perrimon, N. (2005) Genome-wide RNAi screen reveals a specific sensitivity of IRES-containing RNA viruses to host translation inhibition. *Genes Dev.*, **19**, 445–452.

134 Kocks, C., Cho, J.H., Nehme, N., Ulvila, J., Pearson, A.M., Meister, M., Strom, C., Conto, S.L., Hetru, C., Stuart, L.M., Stehle, T., Hoffmann, J.A., Reichhart, J.M., Ferrandon, D., Ramet, M., and Ezekowitz, R.A. (2005) Eater, a transmembrane protein mediating phagocytosis of bacterial pathogens in Drosophila. *Cell*, **123**, 335–346.

135 Philips, J.A., Porto, M.C., Wang, H., Rubin, E.J., and Perrimon, N. (2008) ESCRT factors restrict mycobacterial growth. *Proc. Natl Acad. Sci. USA*, **105**, 3070–3075.

136 Pielage, J.F., Powell, K.R., Kalman, D., and Engel, J.N. (2008) RNAi screen reveals an Abl kinase-dependent host cell pathway involved in *Pseudomonas aeruginosa* internalization. *PLoS Pathog.*, **4**, e1000031.

137 Cherry, S. (2008) Genomic RNAi screening in Drosophila S2 cells: what have we learned about host–pathogen interactions? *Curr. Opin. Microbiol.*, **11**, 262–270.

138 Qin, Q.M., Pei, J., Ancona, V., Shaw, B.D., Ficht, T.A., and de Figueiredo, P. (2008) RNAi screen of endoplasmic reticulum-associated host factors reveals a role for IRE1alpha in supporting *Brucella* replication. *PLoS Pathog.*, **4**, e1000110.

139 Agaisse, H., Burrack, L.S., Philips, J.A., Rubin, E.J., Perrimon, N., and Higgins, D.E. (2005) Genome-wide RNAi screen for host factors required for intracellular bacterial infection. *Science*, **309**, 1248–1251.

140 Cheng, L.W., Viala, J.P., Stuurman, N., Wiedemann, U., Vale, R.D., and Portnoy, D.A. (2005) Use of RNA interference in Drosophila S2 cells to identify host pathways controlling compartmentalization of an intracellular pathogen. *Proc. Natl Acad. Sci. USA*, **102**, 13646–13651.

141 Dorer, M.S., Kirton, D., Bader, J.S., and Isberg, R.R. (2006) RNA interference analysis of *Legionella* in Drosophila cells: exploitation of early secretory apparatus dynamics. *PLoS Pathog.*, **2**, e34.

142 Philips, J.A., Rubin, E.J., and Perrimon, N. (2005) Drosophila RNAi screen reveals CD36 family member required for mycobacterial infection. *Science*, **309**, 1251–1253.

143 Derre, I., Pypaert, M., Dautry-Varsat, A., and Agaisse, H. (2007) RNAi screen in Drosophila cells reveals the involvement of the Tom complex in *Chlamydia* infection. *PLoS Pathog.*, **3**, 1446–1458.

144 Krishnan, M.N., Ng, A., Sukumaran, B., Gilfoy, F.D., Uchil, P.D., Sultana, H., Brass, A.L., Adametz, R., Tsui, M., Qian, F., Montgomery, R.R., Lev, S., Mason, P.W., Koski, R.A., Elledge, S.J., Xavier, R.J., Agaisse, H., and Fikrig, E. (2008) RNA interference screen for human genes associated with West Nile virus infection. *Nature*, **455**, 242–245.

145 Elwell, C., and Engel, J.N. (2005) *Drosophila melanogaster* S2 cells: a model system to study *Chlamydia* interaction with host cells. *Cell Microbiol.*, **7**, 725–739.

146 Rajalingam, K., Sharma, M., Paland, N., Hurwitz, R., Thieck, O., Oswald, M., Machuy, N., and Rudel, T. (2006) IAP-IAP complexes required for apoptosis resistance of *C. trachomatis*-infected cells. *PLoS Pathog.*, **2**, e114.

147 Rajalingam, K., Al Younes, H., Muller, A., Meyer, T.F., Szczepek, A.J., and Rudel, T. (2001) Epithelial cells

infected with *Chlamydophila pneumoniae* (*Chlamydia pneumoniae*) are resistant to apoptosis. *Infect. Immun.*, **69**, 7880–7888.

148 Swanson, K.A., Crane, D.D., and Caldwell, H.D. (2007) *Chlamydia trachomatis* species-specific induction of ezrin tyrosine phosphorylation functions in pathogen entry. *Infect. Immun.*, **75**, 5669–5677.

149 Opitz, B., Forster, S., Hocke, A.C., Maass, M., Schmeck, B., Hippenstiel, S., Suttorp, N., and Krull, M. (2005) Nod1-mediated endothelial cell activation by *Chlamydophila pneumoniae*. *Circ. Res.*, **96**, 319–326.

150 Fellay, J., Shianna, K.V., Ge, D., Colombo, S., Ledergerber, B., Weale, M., Zhang, K., Gumbs, C., Castagna, A., Cossarizza, A., Cozzi-Lepri, A., De Luca, A., Easterbrook, P., Francioli, P., Mallal, S., Martinez-Picado, J., Miro, J.M., Obel, N., Smith, J.P., Wyniger, J., Descombes, P., Antonarakis, S.E., Letvin, N.L., McMichael, A.J., Haynes, B.F., Telenti, A., and Goldstein, D.B. (2007) A whole-genome association study of major determinants for host control of HIV-1. *Science*, **317**, 944–947.

151 Egan, B.J., Marzio, L., O'Connor, H., and O'Morain, C. (2008) Treatment of *Helicobacter pylori* infection. *Helicobacter*, **13**, 35–40.

152 van de Beek, B.D., de Gans, J., McIntyre, P., and Prasad, K. (2004) Steroids in adults with acute bacterial meningitis: a systematic review. *Lancet Infect. Dis.*, **4**, 139–143.

153 Wessler, S., Muenzner, P., Meyer, T.F., and Naumann, M. (2005) The anti-inflammatory compound curcumin inhibits *Neisseria gonorrhoeae*-induced NF-kappaB signaling, release of pro-inflammatorycytokines/chemokines and attenuates adhesion in late infection. *Biol. Chem.*, **386**, 481–490.

154 Tse, A.K., Wan, C.K., Zhu, G.Y., Shen, X.L., Cheung, H.Y., Yang, M., and Fong, W.F. (2007) Magnolol suppresses NF-kappaB activation and NF-kappaB regulated gene expression through inhibition of IkappaB kinase activation. *Mol. Immunol.*, **44**, 2647–2658.

155 Boldicke, T. (2007) Blocking translocation of cell surface molecules from the ER to the cell surface by intracellular antibodies targeted to the ER. *J. Cell Mol. Med.*, **11**, 54–70.

156 Kontermann, R.E. (2004) Intrabodies as therapeutic agents. *Methods*, **34**, 163–170.

157 Lobato, M.N., and Rabbitts, T.H. (2003) Intracellular antibodies and challenges facing their use as therapeutic agents. *Trends Mol. Med.*, **9**, 390–396.

158 Bumcrot, D., Manoharan, M., Koteliansky, V., and Sah, D.W. (2006) RNAi therapeutics: a potential new class of pharmaceutical drugs. *Nat. Chem. Biol.*, **2**, 711–719

159 Kleinman, M.E., Yamada, K., Takeda, A., Chandrasekaran, V., Nozaki, M., Baffi, J.Z., Albuquerque, R.J., Yamasaki, S., Itaya, M., Pan, Y., Appukuttan, B., Gibbs, D., Yang, Z., Kariko, K., Ambati, B.K., Wilgus, T.A., DiPietro, L.A., Sakurai, E., Zhang, K., Smith, J.R., Taylor, E.W., and Ambati, J. (2008) Sequence- and target-independent angiogenesis suppression by siRNA via TLR3. *Nature*, **452**, 591–597.

160 Corey, D.R. (2007) Chemical modification: the key to clinical application of RNA interference? *J. Clin. Invest.*, **117**, 3615–3622.

161 Yamada, T. and Morishita, S., (2005) Accelerated off-target search algorithm for siRNA (2005). *Bioinformatics*, **21**, 1316–1324.

162 de Fougerolles, A.R. (2008) Delivery vehicles for small interfering RNA *in vivo*. *Hum. Gene Ther.*, **19**, 125–132.

163 Dykxhoorn, D.M., and Lieberman, J. (2005) The silent revolution: RNA interference as basic biology, research tool, and therapeutic. *Annu. Rev. Med.*, **56**, 401–423.

164 Behlke, M. (2008) Progress towards *in vivo* use of siRNAs. *Mol. Ther.*, **13**, 644–670.

165 Robbins, M., Judge, A., Ambegia, E., Choi, C., Yaworski, E., Palmer, L., McClintock, K., and Maclachlan, I. (2008) Misinterpreting the therapeutic effects of siRNA caused by immune stimulation. *Hum. Gene Ther.*. doi: 10.1089/hum.2008.131

Part III
Global Approaches of Bacterial Virulence

Bacterial Virulence: Basic Principles, Models and Global Approaches. Edited by Philippe Sansonetti
© 2010 WILEY-VCH Verlag GmbH & Co. KGaA, Weinheim
ISBN: 978-3-527-32326-5

9
The Gut Microbiota and its Contribution to Homeostasis

Pamela Schnupf and Philippe J. Sansonetti

9.1
Introduction

Elucidating the mechanisms by which intestinal pathogens are able to cause disease has been an active field of research for decades. Many of the bacterial factors, cellular pathways and signaling molecules that are involved in disease progression or in combating the pathogen have been elucidated. On the pathogen side, virulence proteins have been identified and functionally characterized at the molecular and cellular levels and *in vivo* where animal models are available. On the host side, we are beginning to have a good understanding of the barriers to gastrointestinal disease progression, ranging from the physical barrier of the intestinal mucosa to the chemical barrier of secreted factors, including bactericidal molecules, and the cellular barriers of both the innate and acquired immune system. Relatively recently, advances in DNA technologies have led to unprecedented insights into the community composition of the resident gastrointestinal microbiota, prompting a surge of interest in the role of the microbiota in health and disease. Importantly in the context of disease, the microbiota is fundamentally important in shaping the inflammatory and immunological response of the host in a way that induces tolerance to the normal resident microbiota yet also primes the host to respond to a breach in its protective barrier. The intestinal mucosa exists in a state of homeostatic balance with its resident microbiota and successful pathogens must therefore disrupt this balance in their favor and subvert the existing defense mechanisms [1]. Thus, to understand how successful pathogens are able to cause disease, we not only have to understand how pathogens are able to manipulate the host but we also have to put these modes of attack into the proper context of the densely-populated gastrointestinal tract, the intrinsic barrier to infection that it provides, and the immunological milieu that this population fosters. Here we highlight some of the new discoveries in the characterization of the gastrointestinal microbiota, the effects of microbial colonization on the host and how the microbiota establishes a symbiotic relationship that protects against pathogens.

Bacterial Virulence: Basic Principles, Models and Global Approaches. Edited by Philippe Sansonetti
© 2010 WILEY-VCH Verlag GmbH & Co. KGaA, Weinheim
ISBN: 978-3-527-32326-5

9.2
Characteristics of the Gastrointestinal Microbiota

The gastrointestinal microbiota is composed of an enormous number and diversity of microorganisms, the vast majority being bacteria although all three domains of life, the Bacteria, Archaea and Eucarya, are represented. Bacterial numbers in the average human gastrointestinal tract are around 10^{14}, or approximately 10 times greater than the number of cells that compose the human body itself [2]. The bacterial load is not uniformly distributed but progressively increases from the proximal to distal end, averaging $10^{2–3}$ bacteria per gram in the proximal ileum and jejenum, $10^{7–8}$ bacteria per gram in the distal ileum and reaching around $10^{11–12}$ bacteria per gram in the colon [3]. This makes the colon the most densely populated microbial niche described to date [4].

9.2.1
Methods to Assess Microbial Diversity

The characterization of the microbial diversity of the gut has benefited from methods and technologies previously used to decipher complex microbial communities in environmental samples (Figure 9.1). Before the genomic revolution, most of our knowledge was derived from bacteriological studies that employed techniques such as microscopy, differential staining, and characterization of the fermentative and biochemical capabilities. These methods relied heavily on the ability to culture the isolates in the laboratory. This requirement proved to be exceedingly limiting as DNA-based technologies that circumvent the need for prior cultivation have revealed that the bacterial diversity in human colon likely includes 500 to 1000 species, the vast majority of which had been and remain uncultivable today [5, 6].

The gold standard for the analysis of the microbial diversity that constitutes a complex community is the phylogenetic analysis of the small subunit (16S) ribosomal RNA gene. This 1500-bp 16S rRNA gene is present in Eucarya, Bacteria and Archaea and is sufficiently conserved to permit reliable sequence alignment between widely different phyla but contains sufficient sequence variability to infer evolutionary relationships [7]. To assess the diversity of the microbial community, 16S rDNA sequences are first grouped into operational taxonomic units (OTUs), or phylotypes, whose levels are by convention defined as >99% similarity for strain level, >97% for species level and >95% for genus level. The intestinal microbiota is by now a sufficiently sampled environment to allow identification of organisms to the genus and even species level. The OTUs are then used to construct phylogenetic trees through pair-wise comparison of all the sequences in a given community. In order to compare different communities, a common phylogenetic tree from all the available sequences is first constructed to which the phylogenetic tree derived from sequence data from each individual community is then compared. The similarity of communities is based on the depth of the branch length they share with the common tree. This ‘UniFrac metric method’ has been highly valu-

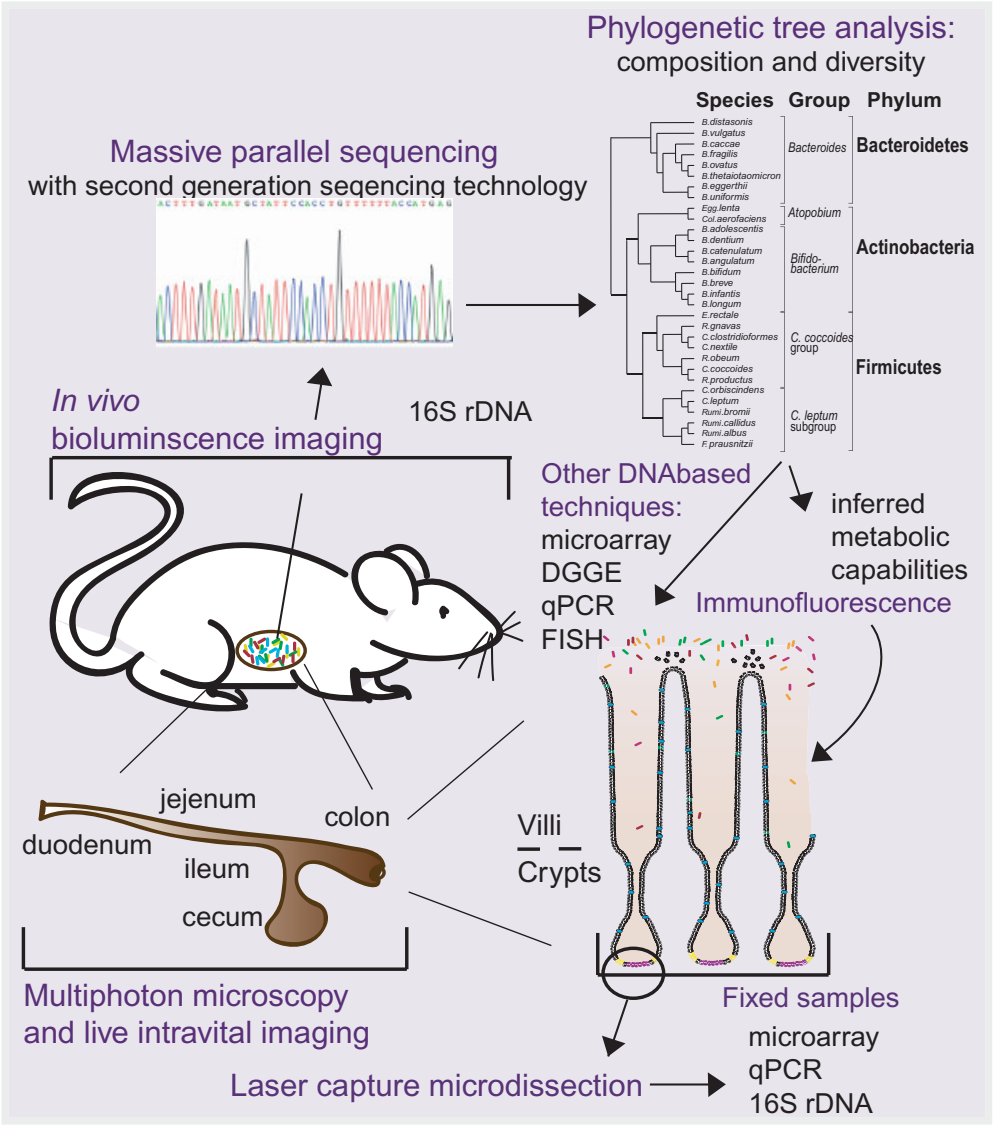


Figure 9.1 Tools available for the analysis of the intestinal microbiota and the host response during steady-state and disease states.

able in comparing the complex microbial community composition of different parts of the gastrointestinal tract such as for example the distal and proximal colon [7]. Phylogenetic identification of the microbial composition also provides, by inference from information available for closely related organisms, a wealth of information on the collective physiology and metabolism of the community,

assuming that the attributed characteristics are conserved in the taxonomic unit. Notably, multiple representatives of the same phylotypes can show a surprising degree of variability in their proteomes, indicating that the total number of genome variants in a community is likely to greatly exceed the number of 16S rDNA-based phylotypes [8]. The physiological and metabolic lifestyles can subsequently be analyzed in more detail by either DNA-based technologies using degenerate primers to inferred factors such as antibiotic resistance genes or metabolic enzymes or by functional testing of inferred metabolic capabilities. The interrogation of the structure, function and metabolic capabilities of the microbial community through culture-independent DNA-based methods is known as metagenomics [9].

Metagenomics has greatly benefited from new high-throughput DNA sequencing technologies. The field of genomics has seen an explosion in both sequencing chemistries and sequencing platforms in recent years that now allows massive parallel sequencing at a greatly increased speed and reduced cost [10]. These second generation sequencing technologies circumvent the need to construct vector libraries by ligating short adaptors to the sheared DNA. The most widely available second generation sequencing methodology is the 454 system by Roche, in which adaptor-flanked DNA is amplified at spatially distinct sites by emulsion PCR [11]. The clonal population of amplified DNA (the amplicon) is captured on 28-um beads and pyrosequenced using primers to the conserved adaptors. Pyrosequencing relies on the use of flow cells that allow the controlled addition and removal of reagents including one of the four unlabeled nucleotides. Upon incorporation of a nucleotide into the growing DNA strand, pyrophosphate is released and used by ATP sulfurylase and luciferase to generate a detectable burst of light. In this manner, the sequence of the growing strand of DNA can be deciphered. While gene-directed analysis of microbial communities has been very informative, the most comprehensive method of studying a microbial community is the shotgun method whereby environmental DNA is isolated *en masse* and used to construct a total DNA library. This metagenomic library that represents the collective genome (the metagenome) of the community can be used for sequence-based or function-driven analysis [12, 13].

The growing availability of cost-effective high-throughput sequencing has led to the rapid expansion of genomic sequence information of the intestinal microbiota and has thereby also propelled other DNA-based analysis tools that rely on previous sequence knowledge, such as quantitative PCR, microarrays, fingerprinting techniques such as denaturing gradient gel electrophoresis (DGGE), and fluorescence *in-situ* hybridization (FISH) microscopy (Figure 9.1) [14]. FISH uses hybridization of DNA probes to a specific bacterial taxon and enables the enumeration of specific bacterial phylotypes and interrogation of the spatial organization of the bacterial phylotype within a mixed community in a heterogenic environment. While current efforts still focus on understanding the diversity of the microbiota present along the gastrointestinstal tract during health and disease states, it will be very interesting to study in more detail how the microbial community is spatially organized at these different times [12, 15].

Tools to dissect changes in the bacterial distribution, abundance and behavior as well as the host response to infection include the rapidly evolving field of imaging technology (Figure 9.1). *In vivo* bioluminescence imaging of pathogens engineered to express luciferase has yielded many and often surprising insights into the infection process such as for example previously missed sites of replication within a host [16]. However, one of the most promising new tools for the detailed study of the gastrointestinal tract is the multiphoton scanning laser microscope. It is powerful enough to penetrate animal tissues up to a working depth of 300 μm, allows dynamic imaging *in vivo* and has already yielded detailed insights into host–pathogen interactions in organs such as the kidneys, lungs, liver, skin and lymph nodes [17, 18]. This emerging field of 'tissue microbiology' involves intravital dynamic imaging of exposed organs from anesthetized animals. In the context of the intestine, this technology has recently led to interesting insights into host processes including the patterns of dendritic cell (DC) sampling of luminal contents. Using transgenic mice with DC-specific expression of green fluorescent protein, DC trans-epithelial cell extrusions can be quantified along the length of the small intestine. The investigators found an increased rate of DC trans-epithelial extensions in the proximal jejenum compared to the terminal ileum and a sampling dependence on the presence of microbial products as well as the particular microbial composition in the intestine [19]. Thus, antibiotic treatment reduced the number of extensions in the proximal part of the small intestine, while introduction of *Salmonella* greatly increased the number of extensions in the terminal section. In addition to monitoring specific fluorescently-labeled host or bacterial cells, fluid-phase and DNA-biding dyes can be injected to visualize in real-time the vasculature and organ architectures [20], while immunohistochemistry of fixed samples can be used to image particular cell types. Furthermore, laser capture microdissection (LCM) of specific microscopic regions of a defined anatomical site can be used for gene expression profiling of the bacteria and/or the host [20, 21]. LCM-mediated gene profiling is highly useful for identifying site-specific differences in the gene regulation of the bacteria or the pathogen and the immune response of the host as well as in defining roles of specific cell types of the host [22, 23].

9.2.2 Diversity of the Gastrointestinal Microbiota

The majority of studies aimed at analyzing the diversity of the human gastrointestinal microbiota use 16S rDNA analysis of stool samples due to the obvious ease of sample acquisition. More recently, sampling of different regions has led to a more detailed view of the microbial diversity at distinct sites along the gastrointestinal tract. In a pioneering study, a 13 335 16S rDNA sequence data set was gathered from biopsies obtained from six regions in the colon and one stool sample from three healthy human adults [5]. This study, and others, revealed that the gastrointestinal environment is highly selective in terms of the overall worldwide microbial diversity. The microbiota of the human intestine contains only nine

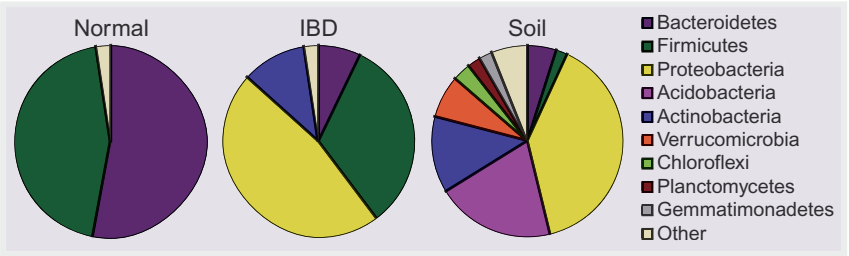


Figure 9.2 Distribution of bacterial phyla in humans and in soil. Comparison of the composition of the bacterial phylotypes present in the soil or the cecum of either healthy humans (Normal) or those with inflammatory bowel disease (IBD). The human data is a compilation of the studies by Eckburg *et al*. [5] and Frank *et al*. [25] as analyzed by Peterson *et al*. [7], while the data for the soil sample was taken from the review by Janssen [24].

of the more than 100 or so phyla, or deep evolutionary lineages in the Bacteria domain and is dominated by only two phyla (Figure 9.2). In comparison, soil samples can yield at least 32 phyla of which nine make up 92% of the sample sequences [24]. Notably, diversity increases greatly in the gastrointestinal tract at lower taxonomic levels. In the human colon, the predominant phyla, identified by 76 and 16% of the sequences, are the low GC, Gram-positive *Firmicutes* and the Gram-negative *Bacteroidetes*, respectively. The remaining 12% belong, in decreasing abundance, to the *Proteobacteria*, *Actinobacteria*, *Fusobacteria* and *Verrucomicrobia* [5]. Other Bacteria that have been reported are *Cyanobacteria*, *Spriochaetes* and *VadinBE97*, while the only phylotype identified from the Archaea domain, which includes at least 13 phyla, was *Methanobrevibacter smithii* [4, 5]. The phylum *Firmicutes* is subdivided into the classes of *Bacilli*, *Clostridia* and *Mollicutes*. In human colon biopsies, 95% of the *Firmicutes* sequences belonged to the strict anaerobic bacteria of the *Clostridia* class, especially those of the clostridial clusters IV, XIVa and XVI that are known to produce butyrate, a short chain fatty acid that serves as a primary energy source for the mucosal epithelium and has important functions for the normal development of the colonic epithelium [5]. Although the phylum composition within the distal gut is largely uniform, the abundance of subgroups within the phylum can change considerably along the gut's cephalocaudal axis. For example, *Streptococcaceae* of the *Bacilli* class made up about 23% of the sequences in tissue samples of the distal small intestine compared to 5% in the colon [25]. Bacterial communities in the intestinal lumen differ from those that are mucosa-associated while the fecal community appears to be composed of members from both niches. However, conflicting reports have been obtained as to whether bacterial communities cluster more closely at mucosal sites along the length of the distal gut or according to a particular biogeographical site across different individuals [5, 26]. The observed difference may depend on the sampling method as well as on prior exposure of the individuals to bowel preparations or antibiotics. What is clearer is that considerable inter-individual variation exists in both environments, especially at the species level.

Unexpectedly, variation in the microbial diversity of the gastrointestinal mucosa at distinct anatomical sites with very different chemical environments is restricted to below the phylum level. The hostile gastric environment of a healthy human stomach was, for example, assumed be rather sterile or at least greatly restricted in bacterial diversity. 16S rDNA analysis, however, found the stomach to be surprisingly rich in bacterial diversity and to overlap extensively with the microbiota of the small intestine, the cecum and the colon [27, 28]. Differences were restricted to the relative contribution of each phylum and the details of the species composition. As in the intestine, *Firmicutes* and *Bacteroidete*s are also abundant phyla in the stomach (20–45% and 10–25%, respectively), but over 80% of the gastric mucosa biopsies (19 out of 23 samples) were dominated (with up to 45% of sequence clones analyzed) by the microaerophilic bacterium *Helicobacter pylori* of the phylum *Proteobacteria*. Notably, the presence of *Helicobacter pylori*, a causative agent of gastric ulcers and stomach cancers, did not greatly affect the remaining composition of the gastric community [27].

Comparisons of the mouse cecal community with the human colonic mucosa and feces, revealed a similar distribution of bacterial diversity at the phylum level. As in humans the dominant divisions were the *Firmicutes* (60–80% of the sequences) and the *Bacteroidetes* (20–40%) and within the *Firmicutes*, the clostridial XIVa cluster was highly represented (75%). Unlike the human colonic samples, the mouse cecum harbored TM7, a previously identified member of the human gingival microbiota, but not *Fusoacteria* [29]. Most studies on the gut microbe community in mammals have been limited to mice and humans although studies using monkeys are emerging. In one large study using rhesus monkeys, sampling from distinct regions of the gut at various times revealed a highly dynamic nature of the gut bacterial composition [30]. Despite extensive characterization, the biodiversity in the gastrointestinal tract remains to be fully explored. Notably, the 16S rDNA primers used in most studies for target DNA amplification are designed to capture Bacteria and Archeae. Yet, a recent survey of the murine intestine revealed a previously unappreciated diversity in fungi, a member of the Eukarya domain [31]. Similarly, with an estimated 1200 viral genomes present in human feces, the diversity of viruses appears to be vast but remains to be fully characterized [32].

9.2.3
Acquisition of the Gastrointestinal Microbiota

The intestinal flora begins to be acquired at birth and progresses until it reaches a more or less stable population that is broadly identifiable in composition as that of a human adult [33]. Several factors have been described that can shape the infant's gastrointestinal microbiota, affecting both the composition and the timing in colonization. These include mode of delivery, i.e. vaginally or cesarean, whether the baby was born preterm of full-term, and whether the infant is formula or breast-fed [29, 34]. While experiments with humans have not strongly supported a kinship effect with regard to the structure of the bacterial community of the

intestinal microbiota, a strong correlation was observed using 16S rDNA sequencing of cecal samples of 25 related and unrelated inbred mice, suggesting that environmental and genetic factors shape the microbial composition [29].

Insights into the developmental pattern of the intestinal microbiota in the first year of life have also been obtained in a microarray-based 16S rDNA analysis of 26 stool samples from 14 full-term human infants [33]. This study revealed great variation in the taxa that dominated the early colonization process, the timing of colonization and the temporal stability of different taxonomic groups. Inter-individual differences were greater than intra-individual differences until about 6 months of age but a general convergence of the community structure to that of a more 'adult-like' composition was observed from 5 days post birth but most strikingly followed the introduction of solid foods [33]. The overall similarity in composition of the adult microbial flora suggests that the microbial community is under environment-specific selective pressure that under normal circumstances restricts the growth of certain bacterial divisions but not others. This notion was supported in a seminal paper that addressed how and to what extent the host gut habitat shapes the microbial community composition [35]. In this study, the intestinal microbiota of conventionally-reared mice and zebrafish were introduced into the gut of germ-free (GF) animals of the opposite species. Upon reciprocal transplantation, the input microbial community underwent a striking rearrangement in both species in its composition, particularly in the dominant bacterial divisions, to more closely mimic that of its native host [35].

9.2.4 Competition within the Intestinal Bacterial Community

Both the host and microbial community shape the microbial composition in the gut. Bacterial and fungal species compete for nutrients as well as space through passive and active means. The relative fitness of a species in a particular environment depends in part on the catabolic capacity and efficiency with which it is able to utilize available nutrients but also depends on its ability to withstand toxic metabolites and inhibitory molecules, particularly bacteriocins or microbially-derived antibiotics that are released by competitors. In addition, a diverse and abundant population of bacteriophages that vary in their host range is present in human feces and likely contributes to the overall composition of the intestinal microbiota [12]. Interestingly, bacteria appear to also elicit the help of their host in combating other intestinal residents. The intestinal mucosa secretes an array of inhibitory molecules including regenerating islet-derived-3γ (RegIIIγ), a C-type lectin that binds tightly to peptidoglycan and exhibits broad antimicrobial activity towards Gram-positive but not Gram-negative bacteria [36]. Gene expression profiling of laser capture-microdissected cecal sections of gnotobiotic mice colonized with the Gram-negative commensal *B. thetaiotamicron* revealed that this species is able to induce a five-fold up-regulation of the RegIIIγ gene as compared to GF control mice. Interestingly, the Gram-positive lactic-acid producing bacterium *B. longum*, a probiotic and a common resident of the distal gut of healthy humans,

was found to be a potent suppressor of RegIIIγ expression, highlighting the intricate methods used by members of the gut microbiota to modulate their environment to confer a competitive advantage on each other [37]. It may thus not be surprising that the presence of a diverse microbiota also fuels the need for microbes to evolve elaborate strategies for immune evasion. For example, *Bacteroides*, the most abundant Gram-negative bacteria in the human intestine, undergo phase variation of the capsular polysaccharides to evade immune surveillance [38]. Acapsular mutants are not more susceptible to cationic detergents, low pH or the presence of bile, but are 30 times more susceptible to killing by complement. Interestingly, although *B. fragilis* has eight capsular polysaccharides, only one polysaccharide is needed by the bacterium to efficiently colonize the intestine of gnotobiotic mice. Yet, the synthesis of multiple, phase-variable polysaccharides is required for the organism to maintain a long-term association in wild-type mice, indicating that the microbiota creates a competitive and complex environment that results in additional pressures for survival [38].

9.3 The Symbiosis of the Gut Microbiota and the Host

The members of the resident microbial population of the gut are generally referred to as commensals. This is misleading, however, for it implies that only one of the partners benefits while the other is unaffected; more accurately, the gut microbiota, or at least members of it, is in a symbiotic relationship with the host whereby both parties benefit from each other. The host intestine provides its microbiota access to a constant and readily fermentable source of carbon and a relatively stable and protected niche. As recognized over 100 years ago by Eli Metchnikoff, the host also profits greatly in terms of gut physiology, overall fitness of the host, and in other, perhaps unexpected, ways [39]. We now know that the complex microbial community in our gastrointestinal tract brings about many beneficial metabolic, physiological, immunological and developmental effects: (i) the microbiota liberates otherwise unavailable carbon sources, expands our own intrinsic catabolic capabilities to harvest and absorb dietary energy, and supplements our nutritional intake with essential microbial-derived metabolites [40]; (ii) colonization by enteric bacteria is essential for normal development of the intestinal epithelium and for the development and homeostatic maintenance of the gut-associated lymphoid tissues (GALT) [41]; and (iii) the microbiota-mediated developmental stimulation of both the innate and acquired immune systems of the gut protects us from infection by enteric pathogens as does the overall competitive ecosystem fostered by the complex community structure of the microbiota [42]. Indeed, due to the extensive benefits that the host derives from its intestinal microbiota, the microbiota can be viewed as an extra organ of the human body that performs important if not critical functions. Nonetheless, the microbiota can also contribute to disease states depending on environmental or host factors while individual species may be neutral, advantageous or harmful to the host depending on whether the

homeostatic balance that normally exists between the host and its microbiota is functional or disrupted.

Dissecting the effects of the gut microbiota on our physiology and health has relied heavily on the comparative analysis of conventionally raised mice, GF or anoxic mice i.e. mice that are raised in a GF environment and lack commensal enteric bacteria, and gnotobiotic or 'conventionalized' mice which are adult GF mice colonized with a defined set of microbes or the distal gut microbiota of conventionally raised mice, respectively [41]. Two enteric bacteria that colonize the gut during weaning and have been singled out as important species that drive the metabolic, developmental and immunological maturation of the gut are the Gram-negative anaerobes *B. thetaiotaomicron* and *Bacteroides fragilis*.

9.3.1 Metabolic Functions of the Microbiota

The gut microbiota facilitates digestion and promotes nutrient uptake and storage by the host. In the absence of intestinal microbes, mice show poor growth, require additional caloric intake in order to maintain their body weight, show intestines congested with an abundance of undigested mucus and require vitamin supplements, especially vitamin K and some B vitamins that are scarce in a regular diet. Colonization of these GF mice with the distal gut microbiota of conventionally raised mice leads to a striking 60% increase in their total body fat content within 2 weeks despite a decrease in their chow consumption [43]. The microbiota brings about these changes through two main mechanisms: fermentation of dietary compounds provides the host with increased quantities of usable energy sources and microbiota-induced stimulation of host signaling pathways leads to enhanced intestinal absorption of nutrients, such as monosaccharides and short-chain fatty acids, and deposition of triglycerides in adipocytes. One important member of the human and mouse gut microflora that produces pronounced changes in the host transcriptional response of genes involved in a wide range of fundamental physiological functions, including nutrient utilization, is *B. thetaiotaomicron*. The conventional microbiota, as well as *B. thetaiotaomicron* alone, direct the host to switch from sialic acid to fucose-terminating glycans [44]. This developmental switch in the host is mediated by changes in the expression pattern of the ileum epithelium which are dependent on the ability of *B. thetaiotaomicron* to use fucose as a carbon source as a fucose isomerase mutant is unable to up-regulate expression of host-derived fucose-terminal glycans upon colonization of GF mice. In addition, *B. thetaiotaomicron* also selectively regulates its carbohydrate foraging activities in response to dietary changes, generally preferring plant polysaccharides but switching to host polysaccharides depending on nutrient availability [45, 46]. The diversity in the types of dietary and host polysaccharides present in the gut makes their digestion a complex process that requires many different types of enzymes and likely depends on the cooperation of members of the microbiota. Interestingly, Sonnenburg *et al.* found, using transcriptional profiling and mass-spectrometric analysis of carbohydrates, that colonizing GF mice with both *B. thetaiotaomicron*

and *B. longum*, a minor member but common probiotic bacterium, expanded the range of polysaccharides targeted for degradation by *B. thetaiotaomicron* [47]. Thus, co-habitation within the gut microbiota can also impact the metabolic repertoire of its constituents.

The number of genes comprising the human intestinal microbiome, as the collective genome of the gut microbiota is known, surpasses the human genome by approximately 100-fold and provides many metabolic capabilities not encoded by the host genome [5, 48]. Large scale metagenomic-based surveys of the metabolic potential of the microbial community in the feces of healthy humans and comparative analysis of the microbiome to the average microbial genome revealed that the human microbiota is significantly enriched in the metabolic pathways of glycans, amino acids, xenobiotics and methanogenesis, as well as the biosynthesis of isoprenoids and vitamins, especially B vitamins [49]. Genes involved in the transport and metabolism of inorganic ions and the biosynthesis, transport and catabolism of secondary metabolite biosynthesis such as antibiotics, pigments and non-ribosomal peptides are underrepresented. The glycobiome of the microbiota encodes at least 81 families of glycoside hydrolases which are mostly absent from the human genome. In particular, the microbial glycobiome encodes enzymes that enable the breakdown of otherwise un-digestible dietary fiber and host-derived glycans, including chondroitin sulfate, mucin, hyaluronate, and heparin [50]. As a comparison, *B. thetaiotaomicron* alone has 64 enzymes able to break down xylan-, pectin-, and arabinose-containing polysaccharides while humans have only one [4]. Fermentation of glycans and dietary fiber are processed mainly to the short-chain fatty acids (SCFAs) acetate, propionate and butyrate, which are efficiently absorbed by the host and account for approximately 10% of the caloric intake from a Western diet [51]. Butyrate is especially important and is the preferred energy source of the colonic mucosa, satisfying up to ~70% of its energy requirement [52]. Concomitant to liberating dietary energy and metabolizing potentially hazardous compounds, the gut microbiota also promotes increased dietary energy utilization by the host by regulating host gene expression to increase the efficiency of monosaccharide and short-chain fatty acid uptake, conversion of SCFAs to more complex lipids in the liver, as well as the subsequent storage of the absorbed energy in adipocytes [4].

9.3.2
Development of the Gut Epithelium Architecture

A large number of studies have shown diverse changes in intestinal morphology, absorptive function, electrolyte handling, bile metabolism, motility, enteroendocrine and exocrine function and production of antimicrobials in the GF state as compared to conventionally-raised mice. In the absence of the gastrointestinal microbial community, the architecture of the intestinal mucosa remains immature and the growth and development of the epithelia are stunted [41, 45]. A particularly noticeable feature is the reduction of proper villus vascularization [53]. During weaning, the villus capillary bed of the intestine of conventionally raised but not

GF, mice, undergoes marked expansion and branching to form a highly interconnected latticework. Strikingly, this developmental deficiency is corrected within 10 days of colonizing an adult GF mouse with a microbiota from conventionally-raised mice or with *B. thetaiotaomicron* alone. How *B. thetaiotaomicron* is able to direct host intestinal vascularization is not well defined but depends on the presence of host Paneth cells in the intestinal crypt and likely on its ability to up-regulate host genes involved in angiogenesis, such as angiogenin-3, from cells within the crypt [53].

9.3.3 Maturation of Lymphoid Tissue

The intestinal microbiota also contributes profoundly to the development of gut lymphoid tissues and the host's innate and acquired immune system. The intestinal mucosa contains a large number of isolated (solitary) lymphoid follicles (ILFs) and Peyer's Patches (PPs), the main organized lymphoid structures of the gut. PPs are mainly present in the distal ileum and in humans contain between five and 200 aggregated lymphoid follicles. PPs increase from around 100 at birth to 250 in the mid-teens and diminish again to approximately 100 between 70 and 95 years of age. In addition, the human gut harbors at least 30 000 ILFs that increase distally in density [54]. In GF mice, these lymphoid structures as well as the draining mesenteric lymph nodes are less frequent, reduced in size, contain fewer cells, and do not form germinal centers i.e. the areas of B cell maturation [41]. Recent studies have shown that sensing of Gram-negative bacterial peptidoglycan by the intracellular innate immune receptor NOD1 is necessary and sufficient to promote the development of ILFs, while maturation of ILFs into B cell clusters is driven by the detection of bacteria through Toll-like receptors [55]. These findings reveal that the maturation of the GALT is not only driven by specific antigens that react with B and T cell receptors, but is also under the control of the innate immune system.

GF mice also show extended changes of the immune cell content in the intestinal lamina propria (LP) and the epithelium. There is a large reduction in $CD4^+$ T cells in the LP, $CD8^+$ T cells in the epithelium and IgA-secreting plasma cells and inducible-nitric-oxide-synthase-positive DCs. The reduced plasma cell count is accompanied by reduced IgA levels in the serum and intestinal secretion [41, 56], while the reduction of $CD4^+$ T-helper cells in the intestinal mucosa is accompanied by an imbalance in regulatory T cells (Tregs) and the potent inflammatory effector Th17 cells. These Th subtypes need to be balanced to avoid disruption of gut homeostasis. In GF animals the Treg/Th17 balance is skewed towards Tregs [57]. In addition, the Th1-subset of inflammatory T helper cells that fight intracellular pathogens and the Th2-subset that help to eliminate extracellular pathogens by directing antibody production is skewed towards Th2 in GF mice [58]. Notably, it was recently discovered that the gut microbial flora also drives the development of a newly discovered innate lymphocyte subtype that is defined by its ability to produces interleukin (IL)-22 [59]. In contrast, macrophage and eosinophil numbers do not seem to be influenced by the microflora [60, 61].

9.3.4
Gut Microbiota Protects against Infection and Transmission of Pathogens

The protective characteristic of the microbiota of a healthy individual, known as 'colonization resistance' or 'microbial interference', is absent under GF conditions and diminished upon disruption and reduction of the microbiota by antibiotic treatment. The intestinal microbiota provides an effective barrier to pathogenic infection and transmissibility. Thus, GF mice are more susceptible than conventionally-raised mice to gastrointestinal infection with for example *Salmonella* spp, *E. coli* and *L. monocytogenes*, while seeding GF mice with members of the intestinal flora provides resistance to infection [62–64]. In addition disruption of the intestinal microbiota by antibiotic treatment enables pathogens and otherwise innocuous, but antibiotic resistant, members of the gut flora to flourish and cause disease [65, 66]. An important example in humans is infection with *C. difficile* as this widespread and normally harmless intestinal anaerobe is a common cause of pseudomembrane colitis in hospital patients undergoing broad-spectrum antibiotic treatment [67]. Indeed, antibiotic treatment prior to infection forms the basis of many animal models of enteric pathogens including *Salmonella* spp., *Streptococcus mutans*, *C. difficile*, *S. flexneri*, and vancomycin-resistent *Enterococcus faecium* [42, 68, 69]. By restricting growth of pathogens, the endogenous microflora also limits their transmission. In a persistently infected mouse model of *Salmonella enterica* serovar *Typhimurium* (*S. typhimurium*) infection, antibiotic treatment increased the number of animals that shed large numbers of bacteria in their feces, leading to rapid and increased transmission of S*almonella* to non-infected cohabiting animals [70]. Beyond protecting the host from pathogens, the microbiota may also protect the host from diarrhea by maintaining the proper osmotic balance in the intestine. Evidence to support this hypothesis was obtained from the analysis of the microbiota from a non-*C. difficile* antibiotic-associated diarrhea (AAD) patient, which revealed a marked reduction in two of the normally predominant groups within the *Firmicutes*, the clostridial clusters IV and XIVa [71]. Many species within these *Clostridia* are efficient in converting fiber and starch into SCFAs, in particular butyrate, which not only serve as important nutrient sources for colonic enterocytes, but also help to maintain osmotic balance [72]. Indeed, clinical studies suggest that intestinal diseases including AAD and *C. difficile*-associated colitis can be treated by seeding the intestine with certain beneficial microbes. These therapeutic microbes known as 'probiotics' include both bacteria and yeast [67, 73].

9.3.5
Gut Microbiota induces Immune Tolerance against Itself, the Host, and Environmental Antigens

Inflammatory bowel disease (IBD) is a group of chronic intestinal diseases characterized by inflammation of the large or small intestine. Although genetic factors have been implicated in the development of IBD, environmental factors also play

a central role as evidenced by a striking rise in its incidence during the course of the twentieth century and the higher prevalence of IBD in industrialized regions [74]. The observation that most animal models for IBD are flora-dependent and that antibiotics show some effectiveness in the treatment of IBD, led to the hypothesis that environmentally-driven changes of the microbiota can cause dysbiosis resulting in a microflora that cannot be tolerated in genetically susceptible hosts [74, 75]. Indeed, remarkable changes in the gut flora from IBD patients have been found, although to date it is not clear whether these alterations are secondary to the onset of disease, perhaps provoked by inflammation, or are responsible for the induction of IBD [7, 25, 76]. However, distinct members of the normal flora in humans and mice have strikingly different effects on the immune system. Probiotic strains are 'symbionts' that protect from bowel inflammation, others are largely ignored by the immune system, and some strains, so-called 'pathobionts', are tolerated in wild-type mice but can induce colitis in susceptible mice strains. It is possible that a dysbalance between symbionts and pathobionts is responsible for the intestinal pathology in IBD [77]. In line with this concept, a recent study revealed that genetically susceptible IBD mice that have developed chronic colitis, can induce intestinal inflammation, albeit less severe, in non-susceptible mice when housed together. This suggests that a dysbiotic flora can be transferred to other hosts, resulting in gut inflammation [78].

It is also interesting to note that the incidence of allergies shows an overwhelming inverse association with infections, vaccinations, and traditional farming environments. Because of these findings the 'hygiene hypothesis' has been formulated. It proposes that reduced exposure to microbiological stimuli results in an increased risk of developing allergy and possibly also autoimmune diseases [79]. Interestingly, changes in the microbiota have been linked to allergenic disorders [80]. Furthermore, probiotic treatment and chronic helminth infections protect from allergies indicating that the gut habitat can also influence immune tolerance to environmental antigens derived from food or pollens [79, 81].

The mechanism of protection by probiotics or symbionts against inflammation remains ill defined but is undoubtedly multi-factorial. Symbionts may be less inflammatory due to differences in their microbe-associated molecular patterns which compete with those of pathobionts [82]. Symbionts may also produce beneficial metabolites such as butyrate which strengthens the epithelial barrier and protects against inflammation [52, 83], or may be composed of factors such as specific capsular polysaccharides that induce a tolerant response in the local or systemic immune system [58]. Strikingly, it has been shown that the single capsular polysaccharide A (PSA) from *B. fragilis* can rectify the lymphocyte imbalance as well as direct the maturation of the lymphoid tissue in GF mice [58]. PSA is a zwitterionic polysaccharides and acts as both a novel T cell antigen and an adjuvant that stimulates innate immune responses by engaging Toll-like receptor 2 (TLR2) [84, 85]. PSA restores the Th2-skewed cytokine profile of GF mice by stimulating

systemic Th1 cytokine production and thereby establishes a healthy Th1/Th2 balance. A disturbed Th1/Th2 is a hallmark of atopic disorders and it has therefore been suggested that PSA is a 'symbiosis factor' that provides a molecular link to the 'hygiene hypothesis' [58]. Furthermore, PSA protects locally from colitis induced by the pathobiont *Helicobacter hepaticus* by inhibiting interleukin-17-producing $CD4^+$T cells (Th17) and inducing interleukin-10-producing $CD4^+$ T cells [86]. Finally, probiotics may shape biofilm formation in the gut, thus changing the spatial compartmentalization of the flora and hindering access of pathobionts and harmful substances to the epithelium [87].

9.3.6
Enteropathogens Exploit Inflammation for their Own Benefit

Inflammation of the gut, whether induced by chemical treatment, infectious diseases or by genetic defects of the host (for example due to a loss of IL-10), is associated with a profound dysbiosis of the colonic microbial community structure [88]. Inflammation results in a decrease in bacterial numbers, a general shift from anaerobic to aerobic bacteria and an overall trend in the reduction of *Bacteroidetes* and a marked increase in the absolute number and proportion of *γ-Proteobacteria*, which include many pathogenic species within the family *Enterobacteriacea* (for example *Escherichia coli*, *Yersinia*, *Salmonella*, *Klebsiella*, *Citrobacter* and *Shigella*). These shifts are not observed when pathogenic colonization of the colon is not associated with inflammation, as exemplified by the murine enteropathogen *Campylobacter* [88]. However, many pathogens use inflammation and the accompanying dysbiosis to their advantage in order to overcome colonization resistance [89]. For example, in streptomycin-treated but not non-treated mice, wild-type *S. typhimurium* infection leads to colonization and colitis. Avirulent *S. typhimurium* mutants that lack the two virulence-associated type III secretion systems are unable to trigger colitis and only transiently colonize the host. Strikingly, this colonization defect can be rescued by concomitant induction of inflammation, either by co-infection with wild-type *S. typhimurium*, by infecting mice suffering from chronic colitis due to the absence of the potent anti-inflammatory cytokine IL-10 (knock-out mice), or by transferring T cells reactive to gut epithelial cells (Villin-$HA^{CL4\text{-}CD8}$) [89]. The concept that inflammation is required to lower the colonization resistance of the gut microbiota is further supported by the finding that avirulent *S. typhimurium* colonize efficiently in the absence of a microbiota despite the absence of inflammation. The molecular mechanisms of this strategy remain unclear. The inflamed gut may offer altered conditions such as changes in the availability of nutrients and adhesion sites that can be exploited by the pathogen but not by the microbiota (the 'food hypothesis') [51]. Alternatively, changes in antimicrobial compounds such as lectins and defensins released by the inflamed tissue can be detrimental to the microbiota but not to the pathogen (the 'differential killing hypothesis') [42].

9.4 Conclusion

The co-habitation of the gut microbiota in the host represents a symbiotic relationship. The bacterial microbiota endows the host with an enormous range of metabolic pathways to degrade luminal dietary substances and, in turn, the gut provides a protected niche for the survival of the co-evolved microbial species that are adapted to the specific environment and its nutritional resources. Both partners have developed mechanisms to guarantee a peaceful alliance and its disturbance may result in inflammatory or metabolic diseases of the host [7, 90] or the loss of competition and, therefore, disappearance of distinct bacterial species [89].

Today, our understanding of the complex human microbiota is largely based on the identification of species by 16S rDNA profiling. However, even the 16S rDNA data are still limited since the inter- and intra-individual diversity and plasticity of the intestinal microbiota remains poorly defined. Some pioneering studies have shown that genetic and environmental factors together with different selective pressures within distinct bowel compartments and changes in lifestyle such as a particular diet, substantially influences the intestinal microbial composition. Yet, we are still far from a comprehensive understanding of the diversity of the normal human gut flora. In addition, disease-associated changes of the microbiota need to be defined in more detail. For humans, this will require well-controlled, population-based cohort studies with large numbers of subjects and longitudinal surveys of patients in different disease states. It will be important to answer whether an abnormal microbiota pattern or single microorganisms alone are sufficient to cause diseases such as IBD or obesity or whether differences in the microbiota composition are mostly the result of secondary changes in the intestinal environment after disease onset [91].

Complete sequencing of selected members of the indigenous microbiota and initial metagenomic analysis has shed light on the functional properties of the microbiome that could previously only be inferred from related organisms. Novel high-throughput sequencing methods and joint research initiatives such as the 'Human microbiome project' will bring forward the analysis of the microbiota pan-genome [92]. To reach an additional level of comprehension of the complex gut flora, investigations will, nevertheless, need to go beyond DNA-level characterization. New technical developments in the area of transcriptional profiling, shotgun proteomics, and bioinformatics will drive this field [7].

The benefits of a deeper understanding of the human microbiome will be multiple. For example, new diagnostic biomarkers of health and disease may be found as well as tools for new treatments, including members of the human microbiota, their metabolic products as well as the chemical messengers they produce. Other important consequences that are anticipated are a more profound insight into the nutritional requirements of humans and new perspectives on contemporary human evolution as the microbiota might reflect a rapidly evolving 'extra-organ' that adapts to changes in the lifestyle of the modern world [92].

Abbreviations

AAD, antibiotic-associated diarrhea; DC, dendritic cell; FISH, fluorescence *in-situ* hybridization; GALT, gut-associated lymphoid tissues; GF, germ-free; IBD, inflammatory bowel disease; IL, interleukin; ILF, isolated lymphoid follicles; LP, lamina propria; LCM, laser capture microdissection; OTU, operational taxonomic units; PPs, Peyer's Patches; RegIIIγ, regenerating islet-derived-IIIγ; SCFA, short-chain fatty acids.

References

1 Sansonetti, P.J., and Di Santo, J.P. (2007) *Immunity*, **26**, 149–161.v

2 Savage, D.C. (1977) *Annu. Rev. Microbiol.*, **31**, 107–133.

3 Neish, A.S. (2009) *Gastroenterology*, **136**, 65–80.

4 Backhed, F., Ley, R.E., Sonnenburg, J.L., Peterson, D.A., and Gordon, J.I. (2005) *Science*, **307**, 1915–1920.

5 Eckburg, P.B., Bik, E.M., Bernstein, C.N., Purdom, E., Dethlefsen, L., Sargent, M., Gill, S.R., Nelson, K.E., and Relman, D.A. (2005) *Science*, **308**, 1635–1638.

6 Hugenholtz, P., Goebel, B.M., and Pace, N.R. (1998) *J. Bacteriol.*, **180**, 4765–4774.

7 Peterson, D.A., Frank, D.N., Pace, N.R., and Gordon, J.I. (2008) *Cell Host Microbe*, **3**, 417–427.

8 Xu, J., Mahowald, M.A., Ley, R.E., Lozupone, C.A., Hamady, M., Martens, E.C., Henrissat, B., Coutinho, P.M., Minx, P., Latreille, P., Cordum, H., Van, B.A., Kim, K., Fulton, R.S., Fulton, L.A., Clifton, S.W., Wilson, R.K., Knight, R.D., and Gordon, J.I. (2007) *PLoS. Biol.*, **5**, e156.

9 Turnbaugh, P.J., and Gordon, J.I. (2008) *Cell*, **134**, 708–713.

10 Shendure, J., and Ji, H. (2008) *Nat. Biotechnol.*, **26**, 1135–1145.

11 Rothberg, J.M., and Leamon, J.H. (2008) *Nat. Biotechnol.*, **26**, 1117–1124.

12 Dethlefsen, L., Eckburg, P.B., Bik, E.M., and Relman, D.A. (2006) *Trends Ecol. Evol.*, **21**, 517–523.

13 Medini, D., Serruto, D., Parkhill, J., Relman, D.A., Donati, C., Moxon, R., Falkow, S., and Rappuoli, R. (2008) *Nat. Rev. Microbiol.*, **6**, 419–430.

14 Ahmed, S., Macfarlane, G.T., Fite, A., McBain, A.J., Gilbert, P., and Macfarlane, S. (2007) *Appl. Environ. Microbiol.*, **73**, 7435–7442.

15 Swidsinski, A., Weber, J., Loening-Baucke, V., Hale, L.P., and Lochs, H. (2005) *J. Clin. Microbiol.*, **43**, 3380–3389.

16 Hardy, J., Francis, K.P., DeBoer, M., Chu, P., Gibbs, K., and Contag, C.H. (2004) *Science*, **303**, 851–853.

17 Konjufca, V., and Miller, M.J. (2009) *Cell Microbiol.*, **11**, 551–559.

18 Hickman, H.D., Bennink, J.R., and Yewdell, J.W. (2009) *Cell Host Microbe*, **5**, 13–21.

19 Chieppa, M., Rescigno, M., Huang, A.Y., and Germain, R.N. (2006) *J. Exp. Med.*, **203**, 2841–2852.

20 Mansson, L.E., Melican, K., Boekel, J., Sandoval, R.M., Hautefort, I., Tanner, G.A., Molitoris, B.A., and Richter-Dahlfors, A. (2007) *Cell Microbiol.*, **9**, 413–424.

21 Melican, K., Boekel, J., Mansson, L.E., Sandoval, R.M., Tanner, G.A., Kallskog, O., Palm, F., Molitoris, B.A., Richter-Dahlfors, A. (2008) *Cell Microbiol.*, **10**, 1987–1998.

22 Hooper, L.V., Stappenbeck, T.S., Hong, C.V., and Gordon, J.I. (2003) *Nat. Immunol.*, **4**, 269–273.

23 Hooper, L.V., Wong, M.H., Thelin, A., Hansson, L., Falk, P.G., and Gordon, J.I. (2001) *Science*, **291**, 881–884.

24 Janssen, P.H. (2006) *Appl. Environ. Microbiol.*, **72**, 1719–1728.

25 Frank, D.N., Feldman, A.L. St Amand, R.A., Boedeker, E.C., Harpaz, N., and

Pace, N.R. (2007) *Proc. Natl Acad. Sci. USA*, **104**, 13780–13785.
26 Dethlefsen, L., Fall-Ngai, M., and Relman, D.A. (2007) *Nature*, **449**, 811–818.
27 Bik, E.M., Eckburg, P.B., Gill, S.R., Nelson, K.E., Purdom, E.A., Francois, F., Perez-Perez, G., Blaser, M.J., and Relman, D.A. (2006) *Proc. Natl Acad. Sci. USA*, **103**, 732–737.
28 Monstein, H.J., Tiveljung, A., Kraft, C.H., Borch, K., and Jonasson, J. (2000) *J. Med. Microbiol.*, **49**, 817–822.
29 Ley, R.E., Backhed, F., Turnbaugh, P., Lozupone, C.A., Knight, R.D., and Gordon, J.I. (2005) *Proc. Natl Acad. Sci. USA*, **102**, 11070–11075.
30 McKenna, P., Hoffmann, C., Minkah, N., Aye, P.P., Lackner, A., Liu, Z., Lozupone, C.A., Hamady, M., Knight, R., and Bushman, F.D. (2008) *PLoS Pathog.*, **4**, e20.
31 Scupham, A.J., Presley, L.L., Wei, B., Bent, E., Griffith, N., McPherson, M., Zhu, F., Oluwadara, O., Rao, N., Braun, J., and Borneman, J. (2006) *Appl. Environ. Microbiol.*, **72**, 793–801.
32 Breitbart, M., Hewson, I., Felts, B., Mahaffy, J.M., Nulton, J., Salamon, P., and Rohwer, F. (2003) *J. Bacteriol.*, **185**, 6220–6223.
33 Palmer, C., Bik, E.M., Digiulio, D.B., Relman, D.A., and Brown, P.O. (2007) *PLoS Biol.*, **5**, e177.
34 Gronlund, M.M., Lehtonen, O.P., Eerola, E., Kero, P., and J. (1999) *Pediatr. Gastroenterol. Nutr.*, **28**, 19–25.
35 Rawls, J.F., Mahowald, M.A., Ley, R.E., and Gordon, J.I. (2006) *Cell*, **127**, 423–433.
36 Cash, H.L., Whitham, C.V., Behrendt, C.L., and Hooper, L.V. (2006) *Science*, **313**, 1126–1130.
37 Sonnenburg, E.D., Sonnenburg, J.L., Manchester, J.K., Hansen, E.E., Chiang, H.C., and Gordon, J.I. (2006) *Proc. Natl Acad. Sci. USA*, **103**, 8834–8839.
38 Coyne, M.J., Chatzidaki-Livanis, M., Paoletti, L.C., and Comstock, L.E. (2008) *Proc. Natl Acad. Sci. USA*, **105**, 13099–13104.
39 Metchnikoff, E., and Mitchell, P.C. (2004) *The Prolongation of Life Optimistic Studies*, Springer, New York.
40 Ley, R.E., Peterson, D.A., and Gordon, J.I. (2006) *Cell*, **124**, 837–848.
41 Smith, K., McCoy, K.D., and Macpherson, A.J. (2007) *Semin. Immunol.*, **19**, 59–69.
42 Stecher, B., and Hardt, W.D. (2008) *Trends Microbiol.*, **16**, 107–114.
43 Backhed, F., Ding, H., Wang, T., Hooper, L.V., Koh, G.Y., Nagy, A., Semenkovich, C.F., and Gordon, J.I. (2004) *Proc. Natl Acad. Sci. USA*, **101**, 15718–15723.
44 Hooper, L.V., Xu, J., Falk, P.G., Midtvedt, T., and Gordon, J.I. (1999) *Proc. Natl Acad. Sci. USA*, **96**, 9833–9838.
45 Hooper, L.V. (2004) *Trends Micro*, **12**, 129–134.
46 Sonnenburg, J.L., Xu, J., Leip, D.D., Chen, C.H., Westover, B.P., Weatherford, J., Buhler, J.D., and Gordon, J.I. (2005) *Science*, **307**, 1955–1959.
47 Sonnenburg, J.L., Chen, C.T., and Gordon, J.I. (2006) *PLoS. Biol.*, **4**, e413.
48 Whitman, W.B., Coleman, D.C., and Wiebe, W.J. (1998) *Proc. Natl Acad. Sci. USA*, **95**, 6578–6583.
49 Gill, S.R., Pop, M., Deboy, R.T., Eckburg, P.B., Turnbaugh, P.J., Samuel, B.S., Gordon, J.I., Relman, D.A., Fraser-Liggett, C.M., and Nelson, K.E. (2006) *Science*, **312**, 1355–1359.
50 Xu, J., Bjursell, M.K., Himrod, J., Deng, S., Carmichael, L.K., Chiang, H.C., Hooper, L.V., and Gordon, J.I. (2003) *Science*, **299**, 2074–2076.
51 Stecher, B., Barthel, M., Schlumberger, M.C., Haberli, L., Rabsch, W., Kremer, M., and Hardt, W.D. (2008) *Cell Microbiol.*, **10**, 1166–1180.
52 Pryde, S.E., Duncan, S.H., Hold, G.L., Stewart, C.S., and Flint, H.J. (2002) *FEMS Microbiol. Lett.*, **217**, 133–139.
53 Stappenbeck, T.S., Hooper, L.V., and Gordon, J.I. (2002) *Proc. Natl Acad. Sci. USA*, **99**, 15451–15455.
54 Brandtzaeg, P., Kiyono, H., Pabst, R., and Russell, M.W. (2008) *Mucosal. Immunol.*, **1**, 31–37.
55 Bouskra, D., Brezillon, C., Berard, M., Werts, C., Varona, R., Boneca, I.G., and Eberl, G. (2008) *Nature*, **456**, 507–510.

56 Tezuka, H., Abe, Y., Iwata, M., Takeuchi, H., Ishikawa, H., Matsushita, M., Shiohara, T., Akira, S., and Ohteki, T. (2007) *Nature*, **448**, 929–933.

57 Ivanov, I.I., Frutos, R.L., Manel, N., Yoshinaga, K., Rifkin, D.B., Sartor, R.B., Finlay, B.B., Littman, D.R. (2008) *Cell Host Microbe*, **4**, 337–349.

58 Mazmanian, S.K., Liu, C.H., Tzianabos, A.O., and Kasper, D.L. (2005) *Cell*, **122**, 107–118.

59 Satoh-Takayama, N., Vosshenrich, C.A., Lesjean-Pottier, S., Sawa, S., Lochner, M., Rattis, F., Mention, J.J., Thiam, K., Cerf-Bensussan, N., Mandelboim, O., Eberl, G., and Di Santo, J.P. (2008) *Immunity*, **29**, 958–970.

60 Mishra, A., Hogan, S.P., Lee, J.J., Foster, P.S., and Rothenberg, M.E. (1999) *J. Clin. Invest.*, **103**, 1719–1727.

61 Pull, S.L., Doherty, J.M., Mills, J.C., Gordon, J.I., and Stappenbeck, T.S. (2005) *Proc. Natl Acad. Sci. USA*, **102**, 99–104.

62 Zachar, Z., and Savage, D.C. (1979) *Infect. Immun.*, **23**, 168–174.

63 Vieira, L.Q., dos Santos, L.M., Neumann, E., Moura, L.N., da Silva, A.P., and Nicoli, J.R. (2008) *J. Clin. Gastroenterol.*, **42** (Suppl. 3 Pt 2), S168–S169.

64 Nardi, R.M., Silva, M.E., Vieira, E.C., Bambirra, E.A., and Nicoli, J.R. (1989) *Braz. J. Med. Biol. Res.*, **22**, 1389–1392.

65 van der Waaij, W.D., Berghuis-de Vries, J.M., Lekkerkerk, L. (1971) *J. Hyg. (Lond.)*, **69**, 405–411.

66 Dethlefsen, L., Huse, S., Sogin, M.L., and Relman, D.A. (2008) *PLoS. Biol.*, **6**, e280.

67 Pillai, A., and Nelson, R.L. (2008) Probiotics for treatment of Clostridium difficile-associated colitis in adults. *Cochrane Database of Systematic Reviews 2008*, Issue 1. Art. No.: CD004611. DOI: 10.1002/14651858.CD004611.pub2.

68 Stecher, B., Hapfelmeier, S., Muller, C., Kremer, M., Stallmach, T., and Hardt, W.D. (2004) *Infect. Immun.*, **72**, 4138–4150.

69 Martino, M.C., Rossi, G., Martini, I., Tattoli, I., Chiavolini, D., Phalipon, A., Sansonetti, P.J., and Bernardini, M.L. (2005) *J. Infect. Dis.*, **192**, 136–148.

70 Lawley, T.D., Bouley, D.M., Hoy, Y.E., Gerke, C., Relman, D.A., and Monack, D.M. (2008) *Infect. Immun.*, **76**, 403–416.

71 Young, V.B., Schmidt, T.M. (2004) *J. Clin. Microbiol.*, **42**, 1203–1206.

72 Topping, D.L., and Clifton, P.M. (2001) *Physiol. Rev.*, **81**, 1031–1064.

73 Sazawal, S., Hiremath, G., Dhingra, U., Malik, P., Deb, S., and Black, R.E. (2006) *Lancet Infect. Dis.*, **6**, 374–382.

74 Koloski, N.A., Bret, L., and Radford-Smith, G. (2008) *World J. Gastroenterol.*, **14**, 165–173.

75 Wirtz, S., and Neurath, M.F. (2007) *Adv. Drug Deliv. Rev.*, **59**, 1073–1083.

76 Scanlan, P.D., Shanahan, F., O'Mahony, C., and Marchesi, J.R. (2006) *Clin. Microbiol.*, **44**, 3980–3988.

77 Round, J.L., and Mazmanian, S.K. (2009) *Nat. Rev. Immunol.*, **9**, 313–323.

78 Garrett, W.S., Lord, G.M., Punit, S., Lugo-Villarino, G., Mazmanian, S.K., Ito, S., Glickman, J.N., and Glimcher, L.H. (2007) *Cell*, **131**, 33–45.

79 Yazdanbakhsh, M., Kremsner, P.G., and van Ree, R., (2002) *Science*, **296**, 490–494.

80 Sepp, E., Julge, K., Vasar, M., Naaber, P., Bjorksten, B., Mikelsaar, M. (1997) *Acta Paediatr.*, **86**, 956–961.

81 Kalliomaki, M., Salminen, S., Arvilommi, H., Kero, P., Koskinen, P., and Isolauri, E. (2001) *Lancet*, **357**, 1076–1079.

82 Munford, R.S. (2008) *Infect. Immun.*, **76**, 454–465.

83 Raqib, R., Sarker, P., Bergman, P., Ara, G., Lindh, M., Sack, D.A., Nasirul Islam, K.M., Gudmundsson, G.H., Andersson, J., and Agerberth, B. (2006) *Proc. Natl Acad. Sci. USA*, **103**, 9178–9183.

84 Wang, Q., McLoughlin, R.M., Cobb, B.A., Charrel-Dennis, M., Zaleski, K.J., Golenbock, D., Tzianabos, A.O., and Kasper, D.L. (2006) *J. Exp. Med.*, **203**, 2853–2863.

85 Cobb, B.A., Wang, Q., Tzianabos, A.O., and Kasper, D.L. (2004) *Cell*, **117**, 677–687.

86 Mazmanian, S.K., Round, J.L., and Kasper, D.L. (2008) *Nature*, **453**, 620–625.

87 Reid, G. (2006) *Trends Microbiol.*, **14**, 348–352.

88 Lupp, C., Robertson, M.L., Wickham, M.E., Sekirov, I., Champion, O.L., Gaynor, E.C., and Finlay, B.B. (2007) *Cell Host Microbe*, **2**, 204.

89 Stecher, B., Robbiani, R., Walker, A.W., Westendorf, A.M., Barthel, M., Kremer, M., Chaffron, S., Macpherson, A.J., Buer, J., Parkhill, J., Dougan, G., von Mering, C., and Hardt, W.D. (2007) *PLoS. Biol.*, **5**, 2177–2189.

90 Turnbaugh, P.J., Ley, R.E., Mahowald, M.A., Magrini, V., Mardis, E.R., and Gordon, J.I. (2006) *Nature*, **444**, 1027–1031.

91 Eckburg, P.B., and Relman, D.A. (2007) *Clin. Infect. Dis.*, **44**, 256–262.

92 Turnbaugh, P.J., Ley, R.E., Hamady, M., Fraser-Liggett, C.M., Knight, R., and Gordon, J.I. (2007) *Nature*, **449**, 804–810.

10
Anatomy of the Gut Barrier and Establishment of Intestinal Homeostasis

Gernot Sellge, Pamela Schnupf, and Philippe J. Sansonetti

10.1
Introduction

The epithelial surface of the human intestine is around 200 m^2, or approximately the size of a tennis court, and is composed of only a single epithelial cell (EC) layer that separates the intestinal lumen from the underlying connective tissue. Given the enormous number and complexity of microbes that inhabit the gastrointestinal (GI) tract and the potential of members of this community to cause disease, it is an incredible challenge to the host to protect itself from injury and pathogenic assault. Many mechanisms that allow the host to shape and control its microbiota have been uncovered. These range from spatial, physical and chemical barriers to immune surveillance. The ever-present threat of microbial intruders necessitates an immunological alertness that allows rapid engagement of the innate and acquired immune system defenses. It is critical, however, that the characteristic inflammatory response triggered by harmful stimuli is tightly controlled to avoid host damage by inappropriate engagement of defenses to non-harmful substances. The long co-evolution of the microbiota and its host has led to a complex cross talk at the molecular and cellular level that puts the host in a state of 'physiological inflammation' [1] whereby the host is primed to initiate a defense response in spite of maintaining immunological tolerance to the microbiota during 'peaceful' times. Indeed, the microbiota contributes to prevent excessive inflammation by stimulating anti-inflammatory responses and both the host and the microbiota cooperate to limit the inflammatory potential of the microbiota itself by shaping the microbiota composition, detoxifying inflammatory antigens and dampening pro-inflammatory cellular signaling.

Here we discuss the anatomical, physiological and immunological properties of the gut barrier, with a focus on the small and large intestine, which control and manipulate the indigenous microbiota and protect against pathogens. We describe the sensing, signaling and response of the host to the microbiota in order to establish gut homeostasis and facilitate a symbiotic relationship. In addition, we give examples of how pathogens disrupt gut barriers and how deregulated cross

Bacterial Virulence: Basic Principles, Models and Global Approaches. Edited by Philippe Sansonetti
© 2010 WILEY-VCH Verlag GmbH & Co. KGaA, Weinheim
ISBN: 978-3-527-32326-5

talk between commensals and the gut immune system might result in inflammatory bowl disease (IBD), a group of inflammatory conditions of mostly the small intestine and colon that are strongly suggested to result from the loss of tolerance to the gut microbiota.

10.2
Anatomical, Physiological and Immunological Properties of the Gut Barrier

The GI tract consists of the oral cavity, pharynx, esophagus, stomach, the small intestine (duodenum, jejunum and ileum), and the large intestine (appendix, cecum, ascending/transverse/descending/sigmoid colon and rectum). The mucosa of each organ is divided into the epithelium, the underlying connective tissue known as the lamina propria (LP) and a thin smooth muscle layer, the muscularis mucosae which is structurally highly specialized to reflect the particular function of the organ. Thus, the stomach is characterized by gastric glands lined with cells that produce mucus, pepsinogen and hydrochloric acid to aid the break down of food while the small intestine and colon contain crypts of Lieberkühn that contain stem cells and specialized mucus and, in case of the small intestine, antimicrobial-secreting cells that are involved in host defense against the increasing density of microbes that colonize the GI tract. As the small intestine is primarily involved in nutrient absorption, the surface of this organ is greatly increased by circular folds, the plicae circulares that are covered by finger-like projections known as villi, which in turn are made up of epithelial cells containing microvilli.

Defenses of the intestinal mucosa against the luminal microbiota can be broadly divided into three categories: (i) pre-epithelial, including the restrictive environment of the lumen, the mucus layer and the aerobic zone above the epithelium; (ii) the intestinal epithelium itself, including the brush border; its properties include acting as a tight barrier, production and secretion of host defense molecules, in addition to its recognition and signaling capacities; and (iii) post-epithelial, including the immune cells of the LP (Figure 10.1). Intestinal immune cells are organized into specific compartments that are divided into 'inductive' and 'effector' sites. The inductive sites in the intestine include the draining lymph nodes (LNs), such as mesenteric LNs (MLNs), as well as constituents of the gut-associated lymphoid tissue (GALT), including the appendix, Peyer's Patches (PPs) and isolated lymphoid follicles (ILFs). Primed GALT-derived lymphocytes re-circulate into the blood via the lymphatic vessels and subsequently migrate to the LP and epithelium that constitute the effector sites [2]. In the LP, primed lymphocytes and a large number

Figure 10.1 The homeostatic 'physiological inflammation' of the intestinal mucosa. During steady-state conditions, a combination of antimicrobial defenses including antimicrobial peptides, mucins and secretory IgA limit access of the microbiota to the epithelium. Antigen and microbial sampling leads to the release of mediators that favor a tolerogenic environment characterized by limited expression of pro-inflammatory cytokines, secretory IgA production and differentiation and maintenance of T regulatory cells.

# of bacteria
antimicrobial factors
Commensal bacteria
Mucus
Villi
Crypts
Differentiation, migration & renewal
# of TLRs
FAE
PP
antimicrobial factors
M cells
Enterocytes
Goblet cells
Enteroendocrine cells
Stem cells
Paneth cells
Apical side
tight junction
Basolateral side
Luminal sampling
Innate and acquired immune defenses limit access of the microbiota to the intestinal epithelium
MAMPS & Quorum sensing molecules
Apical microbial sensing by TLR(s)
TLR(s)
NOD(s)
TLR(s)
Tolerization
M cell
Immune exclusion
Antimicrobial molecules
Secretory IgA
Mucin
TTF3
IgA trans-cytosis
Goblet cell
pIgR
Dimeric IgA
B cell folicular zone
Peyer's Patch
T cell interfolicular zone
Plasma cell
Protective IgA response maintains homeostasis
Mesenteric lymph node
CCR9
α4β7
Mucosal homing of IgA plasmocytes
Dendritic cell
CX3CR1+
BAFF
APRIL
TSLP
TGF-β
IL-10
Macrophage
Immature Dendritic cell
Mucosa-conditioned immune cells with limited expression of pro-inflammatory cytokines
Treg
Suppression of effector T-cells
THelper
IgA response
Production & maintenance of T regulatory lymphocytes achieves sustainable tolerance to the luminal biota
TLR4
MyD88
NOD2
NF-κB
RegIIIγ
Secretory granules
cryptdins
NF-κB
Production and release of antimicrobial factors by Paneth cells protects the crypt
RegIIIγ, cryptdins, angiogenin-4, phospholipase A2, lysozyme, etc.

Figure 10.2 Characteristics of intestinal inflammation in response to a breach in the epithelial barrier. Tissue destruction and microbial sensing induces the expression and secretion of pro-inflammatory cytokines, the production of pro-inflammatory effector cells, molecules involved in antimicrobial defenses such as antimicrobial peptides and mucins, host factors involved in cell protection and wound healing, in addition to chemokines and adhesion molecules for the recruitment of phagocytic immunomodulatory leukocytes.

of innate immune cells function to both assure immune tolerance to the indigenous flora and to combat pathogens (Figures 10.1 and 10.2).

10.2.1
The Intestinal Lumen and the Oxygen Availability at the Tips of the Villi

The bulk of the intestinal microbiota is confined to the luminal space. Within this space, colonization is not unrestricted but rather inter-bacterial competition and environmental conditions limit microbial growth to determine the composition of the microbial community. Characteristics that confer a competitive advantage in this niche include anaerobic fermentation, efficient acquisition and utilization of carbon sources that are non-digestible by the host or are of host origin, as well as resistance to antimicrobial agents released by the host and by members of the microbiota. Spatial separation enables nutrient exchange while preventing direct colonization of the intestinal epithelium and interaction between the potentially toxic agents in the gut lumen and the underlying host tissue [3].

Most members of the intestinal microbiota are anaerobes with no or limited ability to propagate in an aerobic environment, naturally restricting their habitat to the anaerobic environment of the luminal space. In contrast to harmless commensal bacteria, enteropathogenic species are generally at least aerotolerant. Although largely unstudied, access of most of the microbiota to the intestinal epithelium is likely limited by an unfavorable aerobic environment at the lumen/villi interphase as suggested by oxygen-sensing reporter bacteria that can detect an increase in oxygen abundance above the tips of the villi (Martyn, Sansonetti and Tang, unpublished data). Differences in oxygen availability may also affect the virulence potential of enteropathogens such as enterohemorrhagic *E. coli* (*EHEC*) as secretion of virulence factors by the type III secretion machinery is differentially regulated in aerobic and anaerobic conditions [4].

10.2.2
Goblet Cells and the Mucus Layer

The gastrointestinal mucosa is protected by a highly viscous and largely impermeable mucus layer that covers the epithelium and fills the intestinal crypts. This layer is relatively thin in the stomach and proximal small intestine but increases in thickness in the more distal portions and the colon [5]. The mucus layer is predominantly composed of highly glycosylated membrane-bound and soluble

Pathogenic bacteria
Increased production of antimicrobial molecules
Commensal bacteria
Epithelial restitution
TTF3
Mucin release
Tissue destruction and breach of intestinal barrier
Goblet cell
Basolateral microbial sensing by TLRs
IL-22
survival & proliferation
IL-10
Limiting inflammation & protection of mucosa
Differentiation of innate immune cells supporting a pro-inflammatory environment
IL-1β
TNF
IL-6
TLR(s)
CXCL8
Pathogen-specific IgA and igG
Neutrophils
Dendritic cell
Macrophage
IL-23
RORγt
Natural killer-22 cell
CCL20
CCR6
Plasma cell
IL-12
IL-4
TGF-β & RA
+IL-10
TGF-β
IL-6
IL-1β
IL-23
Production of pathogen-specific antibodies
Recruitment of neutrophils, monocytes and immature dendritic cells
T-bet
GATA3
Foxp3
RORγt
TH1
TH2
Treg
TH17
IFN-γ
IL-4
IL-10
TGF-β
IL-17A, IL-17F
IL-21, IL-22
Production of pro-inflammatory effector T cells instead of regulatory T cells involved in tolerance

mucins. The dominant mucin in the intestine, MUC2, is stored and secreted by specialized secretory 'Goblet' cells that are interspersed throughout the epithelial layer and are especially abundant in the colonic crypts. Mucin expression is shaped by the microbiota and physical, chemical or infectious insults lead to the release of copious amounts of mucins from the intracytoplasmic granules of the Goblet cells [6, 7]. In addition to forming a protective protease-resistant matrix, the mucus appears to trap bacteria by mimicking membrane receptors [8, 9]. The mucus thereby competitively inhibits microbes from binding to the epithelial surface and facilitates their elimination via intestinal peristalsis [10]. While the mucus forms a physical protective protease-resistant matrix, it also functions to retain critical host defense molecules including antimicrobial peptides, IgA and the cytoprotective proteins of the Trefoil factor (TFF) family [11–13]. Retention of antimicrobial factors and IgA within the mucus also inhibits bacterial access to the epithelium while allowing bacterial colonization of the intestinal lumen. Similarly, restricted diffusion of TFFs enables these small protease-resistant proteins to act on epithelial cells. Members of the TFF family are produced in a tissue-specific manner by the mucus-producing cells of the GI tract and function to stabilize the mucus layer, protect the mucosa from insults and stimulate epithelial restitution when the integrity of the mucosal barrier has been compromised [13]. In contrast to other wound-healing mechanisms, TFF-mediated repair appears to be TGF-β independent and to occur from the apical area of the epithelium [14].

Thus, under physiologic conditions, the mucus layer efficiently separates the microbiota from the intestinal epithelium. In IBD patients, however, bacteria can be found in close proximity and attached to the epithelium [15], suggesting that defects in the mucus layer promote intestinal inflammation. Indeed, mucin expression is largely altered in IBD patients [16] and mice that lack MUC2 exhibit signs characteristic of distal colitis [17] and develop intestinal adenocarinomas, possibly as a result of chronic inflammation [18]. Furthermore, mice that lack TFF3, the TFF produced by Goblet cells and the main TFF in the colon, are more sensitive to chemical-induced intestinal injury [19].

Due to the protective function of the mucous layer, pathogens have developed strategies to overcome the mucus barrier or interfere with mucin production. For example, the intestinal pathogen *Vibrio cholerae* produces an enzyme that is able to degrade mucins while *Helicobacter pylori*, which colonizes the gastric mucous gel layer, has been shown to reduce mucin exocytosis, cause aberrant expression of the gastric mucins and use its flagella to mobilize itself within the mucus [20, 21].

10.2.3
The Mucosal Epithelium: Physical Barrier and Immune Function

The next line of defense is the single layer of polarized epithelial cells. The mucosal epithelium provides three lines of defense: a structural barrier, apical secretion of antimicrobials, and basolateral secretion of immune regulatory factors. The structural barrier of the epithelium that physically obstructs the translocation of bacteria

from the lumen to the underlying LP is mediated by tight junctions. Tight junctions are formed between adjoining intestinal epithelial cells (IECs) to establish an impermeable cellular barrier that prevents fluid movement across intercellular spaces. Many pathogenic enteric bacteria exploit tight junctions to accomplish their pathogenic strategies by modulating intestinal permeability [22]. Tight junctions are also important for maintaining polarity of the mucosal epithelium by inhibiting lateral diffusion of membrane and membrane-anchored proteins. This polarity enables structural diversification of the intestinal epithelium. IECs feature microvilli only on the apical side that faces the lumen. This so-called brush border maximizes the surface area and contains many digestive enzymes and transporter systems for the breakdown and absorption of dietary compounds. In addition to its physiological role, the structural features of the microvilli, in conjunction with surface glycoproteins, also maintain mucosal integrity by impeding microbial attachment and invasion. In addition, polarized mucosal epithelial cells are uniquely able to distinguish between stimuli from the lumen-facing apical side and the LP-facing basolateral side. This directional sensing appears to be critical for inducing tolerance when a stimulus is presented from the apical side and for mounting an inflammatory response if the barrier function has been breached and the presence of commensals is detected on the basolateral side [23].

Indeed, it is becoming increasingly clear that IECs not only provide a structural barrier but are also key players in the establishment and maintenance of gut homeostasis and in determining the innate and acquired immune response to infection. Epithelial cells sense the external environment and transmit this information to immune cells of the LP by creating an immunomodulatory environment that favors tolerance or inflammation, depending on whether the signals are perceived as innocuous or dangerous. A pathogenic breach of the mucosal epithelium leads to pro-inflammatory cytokine and chemokine production such as interleukin (IL)-6, C chemokine ligand (CCL) 20 and CXCL8 by IEC and recruitment of polymorphonuclear cells (PMNs) and other phagocytes (Figure 10.2). However, under steady-state condition where microbes remain in the luminal space, their presence is not ignored but appears to largely trigger non-inflammatory immune defenses (Figure 10.1). IECs release immune factors, such as TGF-β, thymic stromal lymphopoietin (TSLP), and a proliferation-inducing ligand (APRIL), that recruit macrophages and dendritic cells with a LP-specific 'non-inflammatory' state and help to stimulate differentiation, expansion and/or survival of regulatory T cells (Tregs) and IgA-producing plasma cells [24, 25]. The education of the immune system also relies on the uptake of antigens that can interact with the receptors of the innate and adaptive immune system in the underlying tissue. The epithelium provides this controlled leakiness, mainly through specialized ECs, the 'microfold' (M) cells, but also through normal IECs and the traversal of dendrites from underlying dendritic cells (DCs). Importantly, IECs also determine the environment on the luminal side by producing a broad range of antimicrobial molecules including defensins and cathelicidins and by transporting IgA from the LP to the lumen. Thus, IECs also inhibit bacterial invasion by fostering an inhospitable environment within the mucus layer [23, 26].

10.2.4 Intestinal Crypts, Paneth Cells and Antimicrobials

Intestinal stem cells are located at the base of the crypts and are responsible for the renewal of the intestinal epithelium every few days [27]. New cells differentiate into IECs, Goblet cells, enteroendocrine cells or Paneth cells. With the exception of Paneth cells, which remain at the base of the crypt, these cells migrate up the lateral sides of the crypt to the apical tip of the villi where they undergo programmed cell death and are expelled into the intestinal lumen. The crypt lumen is thought to be a largely sterile environment, thereby protecting the critical multipotent stem cells from microbial challenge and tissue destruction [1]. In a disease state, overgrowth of bacteria leads to 'crypt abscesses', a typical feature of colitis, which is defined by a bacteria-filled crypt lumen with signs of inflammation in the surrounding epithelium and LP.

To assure the 'cleanliness' of the crypt lumen, the host has developed specific defense mechanisms that are different from those present in the villi. For example, the expression of critical host innate immune receptors such as Toll-like receptor (TLR) 2/4 and nucleotide-binding oligomerization domain-containing (NOD) 2 increase with increasing depth of the crypt, allowing for greater sensitivity to bacterial products [28, 29]. In addition, the tolerant state of the immune cells in the underlying LP appears to be less developed in the vicinity of the crypts than in the villi as disruption of junctional adhesions between IECs in the crypts causes intestinal inflammation, whereas it is well tolerated in the villi [30]. An especially critical cell type in the host defense against bacteria in the crypt lumen is the Paneth cell. Paneth cells are specialized secretory cells located at the base of the crypts of Lieberkühn in the small intestine that secrete and store a wide variety of antimicrobials in the apical granules (Figure 10.1) [31].

Antimicrobials are a large family of various peptides or proteins that generally target essential cell wall structures of microorganisms. Antimicrobials encompass enzymes that kill bacteria by enzymatic attack on microbial cell walls (e.g. lysozyme and secretory phospholipase A2), and pore-forming peptides that disrupt bacterial cell membranes (α-defensins or cryptdins in mice, β-defensins, and cathelicidins) [32]. In addition, there is a growing group of antimicrobials with as yet poorly defined mechanisms, such as angiogenin (Ang)-4 (in mice, similar to Ang-1 in humans) which is a member of the ribonuclease family [33], and specific C-type lectins (RegIIIγ in mice and HIP/PAP in humans) that bind peptidoglycans [34]. Antimicrobial peptides display selective microbicidal activity towards Gram-positive and -negative bacteria, fungi, viruses and protozoa. The range of antimicrobial activity is, however, not easily predicted as even two highly related bacteria can be differentially susceptible to particular antimicrobials, and, conversely, structurally similar antimicrobial peptides act against very different species. For example, *L. monocytogenes* is highly susceptible *in vitro* to Ang-4, while the non-pathogenic species *Listeria innocua* is resistant to up to 10 times higher concentrations of Ang-4 [33]. Conversely, the abundant human α-defensin HD5 is microbicidal *in vitro* towards many bacterial species and the fungus *Candida albicans* while the

structurally similar HD6 defensin shows poor microbicidal activity towards these microbes [31].

Antimicrobials are expressed along the GI tract, although their pattern of expression varies at different anatomical locations. Paneth cells are the most important producers, as they produce all of the proteins described above, except for β-defensins and cathelicidins, which are produced by myeloid cells and IECs in the small and large intestine. The majority of α-defensins, and cathelicidins, lysozyme, and secretory phospholipase A2 are synthesized constitutively and not in response to microbial sensing. Instead, they are stored in the intracellular granules of Paneth cells and released upon antigen stimulation. This rapid secretion occurs in response to Gram-negative and -positive bacteria, lipopolysaccharide, lipoteichoic acid, lipid A and muramyl dipeptide [35]. However, the production of RegIIIγ, Ang-4 and certain α- and β-defensins are under the control of the microbiota and require signals delivered via TLRs and NOD2 [29, 32, 36] (Figure 10.1). α-Defensins, whose activity accounts for 70% of the bactericidal activity in Paneth cells [35], are under additional regulation as they are expressed as inactive prepropeptides and their activation requires intracellular (in mice via the metalloprotease matrilysin-7) or extracellular (in humans via trypsin) cleavage of the inhibitory pro segment. Interestingly, commensals appear to stimulate production of antimicrobials for their own benefit. For example, the Gram-negative strain *B. thetaiotamicron,* but not the Gram-positive strain *Bifidobacterium longum*, induces the expression of RegIIIγ. However, RegIIIγ is only bactericidal against Gram-positive bacteria, suggesting that its production provides a competitive advantage to *B. thetaiotamicron* [34, 37]. Pathogens have developed strategies to down-regulate antimicrobials, as has been shown for *S. flexneri*, which represses through the action of type III secretion effectors, the production of β-defensin 3 and the cathelicidin LL37 in IECs [38].

Host-derived antimicrobials contribute to the maintenance of intestinal homeostasis by protecting the host against commensal-induced damage, inflammation and pathogenic challenge. Consequently, decreased or unbalanced production of antimicrobial peptides has been linked to disease states. For example, loss of NF-κB-dependent expression of cryptdins and mouse β-defensin-3 in IECs in the murine colon was linked to compromised integrity of the colonic epithelium, translocation of luminal bacteria into the mucosa and concomitant initiation of the inflammatory response [39]. In addition, mice with defects in cryptdin activity due to the deletion of matrilysin-7 are defective in bacterial clearance and more susceptible to infection by *Salmonella enterica* serovar *Typhimurium* [40] while NOD2 knock-out mice that display reduced cryptdin expression are more susceptible to infection by *L. monocytogenes* [29]. It has also been suggested recently that nosocomial infections can become established after broad-spectrum antibiotic therapy because of a change in the expression level of certain antimicrobials. After antibiotic treatment, mice showed a largely reduced microbiota resulting in diminished TLR-dependent stimulation of RegIIIγ production in Paneth cells, which in turn enabled vancomycin-resistant *Enterococci* to establish an infection in the host [41]. Conversely, when mice were engineered to express the most abundant human

α-defensin, HD5, in their Paneth cells, they became more resistant to oral infection with *S. typhimurium* [42].

Dysregulated production of antimicrobials might also play an important role in IBD. The finding that NOD2 governs expression of a key subset of α-defensins in mice [29] suggests the possibility that lowered α-defensin expression could be associated with Crohn's disease, one type of IBD that is linked to loss-of function mutations in NOD2. Indeed, analysis of α-defensin expression in Crohn's disease (CD) patients, in particular those harboring NOD2 mutations, showed reduced α-defensin expression [43]. These findings suggest a model in which NOD2 mutations result in reduced α-defensin production and a subsequent defective defense against the normal flora. Conversely, a recent elegant study using *Drosophila* showed that the up-regulation of antimicrobial networks can also result in pathological effects [44]. In this study, RNA interference of a negative regulator of the NF-κB-dependent antimicrobial gene expression resulted in the uncontrolled up-regulation of a subgroup of antimicrobials that led to a selective deletion of certain (sensitive) members of the microbiota and overgrowth of other (non-sensitive) strains. The resulting dysbiosis resulted in a loss of tolerance to the microbiota, gut cell apoptosis and host cell death.

10.2.5
Innate Immune Cells of the Lamina Propria

The LP of the small and large intestine contains a large number of different innate immune cells such as macrophages (Mϕs), DCs, mast cells, natural killer (NK) cells, and eosinophils, while PMNs and basophils are largely absent under steady-state conditions but enter the tissue during the course of acute inflammation (PMNs) or helminth infections (basophils1) (Figures 10.1 and 10.2) [45]. The phenotype of the innate immune cells of the GI tract is distinct from their relatives in other tissues and follows the concept of 'armed peace' of the intestinal immune system [1]. Indeed, signs of both permanent activation and tolerating mechanisms can be found.

Mϕs are well described professional phagocytes with pro-inflammatory and antigen-presenting functions. Mϕs are derived from blood monocytes which can differentiate into specialized Mϕs as they enter the tissues, using a process dependent on the specific environment of the target tissue. This tissue-specific activation leads to Mϕs with distinct morphological and functional characteristics in different anatomical sites (e.g. Kupfer cells in the liver, osteoclasts in bones, and microglia in the nervous system). Intestinal Mϕs, in contrast to monocytes, lack expression of several co-stimulatory molecules, cytokine receptors, adhesion molecules, complement receptors, Fc receptors, and TLRs. Hence, intestinal Mϕs are hypo-reactive to Fc receptor cross-linking and TLR stimulation [46–49]. Yet, surprisingly, despite this general 'non-inflammatory' status, intestinal Mϕs retain avid phagocytic and bactericidal activity for commensals and pathogens and are therefore perfectly equipped to act as quiescent sweepers against barrier-breaching commensals [46, 50]. Notably, resisting macrophage killing is a common strategy used

by invasive pathogens to promote their virulence although very different tactics are used: *Salmonella* survives and replicates in a modified phagosome called the *Salmonella*-containing vacuole; *L. monocytogens* escapes the phagosomal compartment and utilizes the protected niche of the Mϕ cytosol for replication and dissemination in a Trojan horse-style strategy; *Yersinia* spp. evade Mϕs altogether by injecting bacterial proteins with anti-phagocytic properties into the Mϕs and triggering macrophage apoptosis; while *Shigella* is phagocytosed but rapidly kills macrophages with concomitant release of pro-inflammatory mediators [1]. The role of Mϕs in maintaining homeostasis is further supported by the finding that intestinal Mϕs can induce the differentiation of regulatory but not effector T cells [48]. The differentiation of monocytes to intestinal Mϕs is constrained by factors derived from local epithelial, stromal, and immune cells such as IL-10 and TGF-β [46, 48] and is suggested to be independent of the commensal flora [51, 52]. Whether resident intestinal Mϕ can regain their pro-inflammatory phenotype in the course of infections or other inflammatory situations is unclear. However, the recruitment of monocytes from the blood to the gut is required for optimal immune defense against pathogens such *Toxoplasma gondii* [53]. Apart from their role in immune defense, intestinal Mϕs are necessary for epithelial renewal. After injury, Mϕs are recruited to the base of the crypt and release factors supporting epithelial growth [52].

Like Mϕs, resident intestinal mast cells are hypoactive when isolated from the human gut, as they are unresponsive to IgE-crosslinking, TLR ligands and Gram-negative bacteria [54, 55]. However, they store large amounts of pro-inflammatory mediators, including cytokines such as Tumor necrosis factor (TNF), even in the homeostatic situation [56]. Mast cell-derived TNF is required for optimal protection against bacterial lung infections and polymicrobial sepsis [57, 58]. This suggests that intestinal mast cells are quiescent during steady-state confrontations with incoming commensals but may react during pathogenic bacterial invasion, e.g. through activation by toxins or cell lysis [54].

Eosinophils are granulocytes that account for only 1 to 3% of peripheral blood leukocytes and infiltrate various tissues in the course of allergic disorders, asthma, IBD and certain infectious diseases, in particular helminth infections [59, 60]. Under physiological conditions, significant levels of tissue eosinophils can only be detected in lymphoid organs such as the spleen, peripheral LNs, and the thymus and in the LP of the GI tract. Notably, only GI eosinophils show characteristics of permanent degranulation, a sign of activation, under homeostatic conditions [61]. Eosinophils are activated by different inflammatory mediators (e.g. the anaphylatoxins C3a and C5a), microbial products (e.g. formyl-met-leu-phe (fMLP) and TLR7/8 ligands), or Fc receptors, that lead to the release a large array of stored (within granules) and *de-novo* synthesized factors, such cytokines (including pro- and anti-inflammatory), eicosanoids, and largely eosinophils-specific cationic proteins with anti-helminthic, anti-bacterial, and anti-viral functions. Although *in vivo* functional data for the GI tract, apart from their protective role in helminth infections, are largely missing, the characteristics of eosinophils described above strongly suggest that they have important functions in host defense and homeostasis [59].

NK cells are lymphocytes of the innate immune system that lack somatically rearranged antigen-specific cell surface receptors. Instead, the combinatorial engagement of germline-encoded activating and inhibitory receptors 'tune' NK cell cytotoxicity and the secretion of cytokines and chemokines. Recent observations have shown that, apart from classical NK cells, the gut also contains another type of innate lymphocyte (NK-22 in humans or Nkp46$^+$ RORγt$^+$ cells in mice) that shares some phenotypical characteristics with NK cells. However, this lymphocyte subtype lacks typical NK functions but instead produces IL-22, a cytokine that regulates epithelial proliferation and expression of antimicrobials. NK-22/ Nkp46$^+$ RORγt$^+$ cells have thus been linked to intestinal homeostasis and immune defense against pathogens. These cells could only be found in the tonsils, GALT, and intestinal LP and their development was shown to be dependent on the presence of the microbiota [62, 63].

10.2.6
Antigen Sampling in the Gut

The gut epithelium forms a tight barrier to contain the substantial bulk of food and indigenous microbes present in the lumen. However, soluble antigens such as non- or partially-digested food proteins and bacterial products (e.g. peptidoglycans) as well as whole commensal bacteria can be detected in the mucosa and draining LNs while soluble antigens can also be found in the blood and may reach other tissues [50, 64–66]. During intestinal steady-state conditions, this antigen uptake is tightly regulated and can occur via different routes including passage through IECs and M cells or through direct uptake by transepithelial dendrites of DCs (Figure 10.1). Future work will need to address the relative contribution of specific luminal antigen acquisition routes that are linked to the induction and constant renewal of tolerance and to the development of protective immune responses [67].

M cells are considered to be the most important antigen samplers. They are predominantly localized in the follicle-associated epithelium (FAE), the specialized epithelial regions that cover lymphoid follicles. Fewer M cells are found interspersed throughout the intestinal villi where they are independent of FAE (villous M cells) [68]. Intestinal M cells differ from adjacent IECs by the absence of a typical brush border and the presence of variable microfolds, a thin cytoplasm and an intraepithelial pocket that contains lymphocytes and antigen-presenting cells. M cells very efficiently translocate and deliver luminal antigens (soluble or particulate, including whole bacteria) to antigen-presenting cells located in the subepithelial regions [69]. Notably, many enteric pathogens including *Yersinia*, *Shigella* and *Salmonella* spp. exploit M cell transport as a portal to invade the intestinal mucosa [1].

Antigens can also reach the basolateral region of the gut by the transcellular passage of normal IECs and/or via paracellular flux although the latter route is most likely confined to the inflamed mucosa (Figure 10.2). IEC passage occurs via the endocytic pathway, eventually leading to the formation of MHC–antigen com-

plexes that will either be displayed on the basolateral membrane and made accessible to adjacent lymphocytes or will be released on exosomes [64]. In addition, the neonatal Fc receptor (FcRn) allows a bi-directional passage of IgG through the intestinal epithelium. Secreted IgG binds to cognate luminal antigens (including whole bacteria) to form IgG/antigen complexes that are recycled by FcRn receptors and delivered to LP antigen-presenting cells. This pathway has been suggested to be of particular importance for the development of adaptive immune responses in mice against epithelium-associated pathogens such as *Citrobacter rodentium* [70, 71].

DCs are the most important antigen-presenting cells which phagocytose and process antigens that have reached the LP but also play an important role in monitoring the luminal milieu via trans-epithelial extensions. It has been shown that DCs sample luminal microbes by opening tight junctions between adjoining ECs and extending projections across the epithelial layer. The integrity of the epithelial barrier is maintained throughout this process as DCs form tight junction-like structures with ECs by expressing tight junction proteins such as occludin, claudin I and zonula occludens I [72]. Trans-epithelial dendrite formation of DCs is mainly restricted to the small intestine and is driven by epithelial TLR engagement [73]. In addition, DCs require expression of CX_3CR1, the receptor of the membrane-bound chemokine CX_3CL1 expressed on the basolateral side of IECs, for luminal sampling and full antibacterial defense against entero-invasive pathogens [74]. DCs constitutively traffic to the T cell areas in the GALT and draining LNs to present sampled antigens derived from food, bacteria, and apoptotic IECs to T and B cells [75]. In contrast to Mϕs that kill the phagocytosed bacteria, intestinal DCs can support small numbers of live commensals for several days. These commensal-loaded DCs migrate as far as the mesenteric lymph nodes but not further to other systemic lymphoid compartments such as the spleen [50]. The gate-keeping function of the MLNs thereby restricts migration of commensal-activated DCs to within the intestine and permits local mucosal immune responses against the intestinal biota to develop while avoiding the generation of systemic immunity which is required exclusively when pathogens breach the intestinal barrier and migrate beyond the intestinal mucosa.

DCs are a heterogeneous population characterized by a remarkable plasticity and can be divided into conventional DCs ($CD11c^{high}$) and plasmacytoid DCs ($B220^{+}CD11c^{low}$). Conventional DCs are the main subtype in the intestine and can be further subcategorized by the expression of CD11b, CD8, CD103, chemokine receptors, or inducible-nitric-oxide-synthase (iNOS). Intestinal DCs are in general considered to be less pro-inflammatory and more tolerogenic than their splenic counterparts [76]. The conditioning of intestinal DCs is effected by factors derived from food (Vitamin A as a precursor of retinoic acid (RA)), commensals (butyrate or indirectly e.g. by the activation of TSLP production by IECs), IECs (TGF-β, TSLP, RA), stromal cells (TGF-β, RA), and other immune cells (IL-10, TGF) [75, 77–80]. This tolerizing microenvironment changes during the course of inflammation and leads to the production of pro-inflammatory and chemotactic factors by IECs and immune cells, resulting in the recruitment of new DCs. It remains

unclear, however, whether the generation of a protective effector immune response in the gut is generally mediated by the recruitment of DCs from the blood that have not previously been subject to LP conditioning or by the activation of specific subtypes of pre-existing DCs (e.g. $CD103^-$ in MLNs or $CD11b^+$ in the LP) via, for example, TLR engagement. There is evidence for both scenarios and most likely the two pathways act in parallel [75, 81, 82].

10.2.7 The Protective IgA Response

The commensal microbiota triggers the production of specific IgA, the major immunoglobulin secreted by LP plasma cells into the intestinal lumen. It is estimated that over 80% of all plasma cells reside in the gut LP and that they produce more IgA (40–60 mg/kg/day) than all other isotypes combined. Circulating IgA is mainly monomeric whereas secretory IgA (SIgA) forms dimers or polymers that originate from the interaction with the J chain, a polypeptide synthesized by plasma cells. The J chain also interacts with the polymeric immunoglobulin receptor (pIgR), which is responsible for the transcytosis of SIgA across IECs. In contrast to mice, there are two subclasses of human IgA, IgA1 and IgA2. Whereas systemic plasma cells produce mostly IgA1, IgA2 is very abundant in the intestine and is more resistant than IgA1 to degradation by bacterial proteases [83]. Notably, microbiota-specific IgG is not formed under physiological conditions but can be found in IBD patients, indicating the loss of tolerance to the commensal flora [84].

IgA functions by binding its antigen-specific variable side to soluble or cell-bound antigens (proteins or carbohydrates). In general, IgA mediates 'immune exclusion' because it prevents the contact of antigens with adaptive and innate immune receptors and does not cause inflammation itself due to its inability to fix and activate the complement cascade. The activating Fcα receptor (CD89) is scarcely expressed on intestinal immune cells, suggesting that its function is confined to the systemic compartment [47]. In the LP, IgA binds bacteria or their products and shuttles them to the lumen by pIgR-mediated transport. IgA in transit through the epithelium can inhibit virus production or neutralize pro-inflammatory microbe-associated molecular patterns (MAMPs). In the lumen, it neutralizes toxins and anchors commensal bacteria to the mucus, thereby impeding bacterial entry into the underlying intestinal mucosa. At the same time SIgA facilitates controlled uptake of opsonized antigens by M cells via a receptor-mediated transport [25, 85]. Different animal models have shown that IgA limits bacterial translocation [50], inhibits the innate immune response to commensals [86], and protects against invading pathogens [87]. In humans, some patients with selective IgA deficiencies suffer from gastrointestinal and pulmonary infections, allergies, and also auto-immune diseases [88]. Recent studies have shown that IgA also determines the composition of the intestinal flora at the species levels, e.g. the aberrant expansion of segmented filamentous bacteria in mice with IgA-deficiencies [89]. Furthermore, specific IgA induces down-regulation of its cognate epitopes on the surface of commensals [86] highlighting the selective pressure IgA

exerts on the gut microbiota. IgA is preferentially produced against bacteria that continue to stimulate the innate system. A solely non-selective response would remove these species in addition to other non-specific species in order to maintain the immunological homeostasis. Contrarily, a second-line epitope-specific response provided only by the adaptive immune system induces diversification of surface antigens that lead to tolerance and removes only those phylotypes that cannot adapt to the host's immune system. This type of selective pressure may provide one explanation for the remarkable strain-level diversity in the gut microbial community [86].

Growing evidence indicates that B cells use different developmental pathways to produce high-affinity and low-affinity IgA. The high-affinity pathway is T cell-dependent and serves to neutralize microbial toxins and pathogens, while the low-affinity pathway, which produces IgA with broad specificity, is T cell-independent and appears to prevent commensal bacteria from breaching the mucosal surface [83]. The maturation of B cells to IgA-producing plasma cells requires several consecutive steps. Immature B cells in the bone marrow generate antigen recognition diversity through somatic gene segment recombination, which is initiated by an antigen-independent mechanism. These immature B cells migrate to secondary lymphoid organs such as the GALT where the antigen-dependent phase of B-cell development takes place. B-cell activation is initiated following engagement of the clonotypic B-cell receptor by specific soluble antigens or membrane-associated antigens presented by DCs. Upon antigen stimulation, mature B cells diversify their antibody repertoire by class switching from IgM and IgD to IgG, IgA, or IgE and somatic hypermutation to create high-affinity antibodies. These processes take place in the germinal centers of secondary lymphoid follicles and are dependent on cognate interactions between antigen-specific B cells and $CD4^+$ T helper (Th) cells. Th cells provide the two major signals responsible for the class switch reaction (CSR), the CD40 ligand (CD40L), which engages CD40 on B cells, and cytokines that direct the class switch reaction either to IgG, IgA, or IgE. Gut T cells are conditioned to produce cytokines such as TGF-β, which together with IL-6, IL-10 and RA produced by DCs and stromal cells, facilitate preferential CSR of B cells from IgM to IgA. In addition, the GALT microenvironment imprints lymphocytes with gut-seeking properties. For example, RA induces up-regulation of gut-homing receptors such as CCR9 and α4β7 integrins on B cells and also T cells (Figure 10.1). IgA^+ effector B cells migrate to the LP where they differentiate into IgA-secreting plasma cells [25, 90]. However, specialized B cell subsets (e.g. B1 cells in mice) can produce IgA in a T cell- and CD40L-independent manner. Patients with deficiencies in $CD4^+$ T cells (due to HIV infection) or CD40 [25], as well as mice lacking T cells, retain intestinal IgA, albeit at reduced levels, that maintain binding specificities to commensals [91]. Although still debated, T cell-independent class switching might take place in ILFs and in the LP and is dependent on two B cells-stimulating factors structurally and functionally similar to CD40L, B cell-activating factor (BAFF) and APRIL. It has been shown that in response to microbiota-derived signals, APRIL is released by IECs and both APRIL and BAFF are produced by a specific DC subset (TNF/iNOS-producing [Tip]DCs) via an

iNOS-dependent pathway [90, 92]. This more primitive and perhaps evolutionarily older 'innate' SIgA system is considered to be the front line defense against the induction of autoimmunity and bacterial invasion and may also be important in providing herd immunity by rendering fecal bacteria less invasive [25, 93, 94].

10.2.8
The Balance between Regulatory and Effector T Cells in the Gut

The ability to distinguish between self and non-self represents a key feature of the immune system. However, the differentiation between the dangerous non-self, such as pathogens, and the harmless, or better useful, non-self, such as commensals or food, is an additional task. The innate immune system seems to be central in the initial decision-making process as to whether antigens should be tolerated or not due to its ability to generate a tolerogenic environment when it encounters harmless commensals and food antigens and to generate a pro-inflammatory response upon infection or injury. T cells then 'read' the information derived from the innate immune cells to develop antigen-specific regulatory or effector T-cell responses as required [95]. Mucosal immune adaptation to commensals is not purely dependent on the adaptive immune response. Animals with selective or combined deficiencies of the B or T cells are able to live with an intestinal bacterial flora without adverse effects in pathogen-free facilities. However, they exhibit increased levels of commensals translocation within the mucosa and the draining LNs and maybe more importantly, are more susceptible to many pathogens [96]. Notably, mice that selectively lack Tregs develop chronic intestinal inflammation as a result of an overreaction of their effector T (or Th) cells to the microbiota. These findings suggest that the major task of Tregs is to control the effector T cell compartment rather than the innate immune system and that the equilibrium between effector T cells and Treg at the intestinal mucosa is crucial to maintaining the immunological homeostasis [97].

T cell development also occurs in several stages starting in the thymus where the rearrangement of the T cell receptor (TCR) and subsequent positive and negative selection occurs. Naïve antigen-specific T cells migrate to secondary lymphoid tissues where they are activated by specific antigens presented by antigen-presenting cells. This interaction leads to their expansion, maturation and subsequent migration to effector sites such as the LP and epithelium of the GI tract. T cells comprise $CD4^+$ subsets, cytotoxic T cells ($CD8^+$), and non-conventional T-cell subsets (e.g. TCRγδ, NKT, or mucosal-associated invariant T cells) whose ontogeny is poorly defined but differs from that of conventional T cells [98].

Cytotoxic T cells and non-conventional T cells are largely interspersed among IECs in both the small and large intestine. The frequency of occurrence of the intraepithelial lymphocyte (IEL) varies, with one IEL for every 4–10 IECs in the small intestine and one for every 30–50 IECs in the large intestine [99]. The non-conventional or 'type b' IELs contain TCRαβ- as well as TCRγδ-expressing T cells that do not express the conventional TCR co-receptors and are thought to be largely self-specific. For example, IELs can use both γδTCR and NKG2D, a NK receptor

expressed on a subpopulation of IELs, to recognize damaged or stressed IECs through MICA/B (MHC class I chain-related gene A/B) [100, 101]. Although the functional role of IELs remains ill defined, it is evident that they are involved in the first line immune surveillance system and play a pivotal role in immune defense, immune regulation, and epithelial repair. Upon infection, IELs provide protection through antigen-specific cytotoxic activity and cytokine production such as interferon (IFN)-γ. Regulatory functions and epithelial repair are suggested to be mediated by their ability to produce IL-10, TGF-β, and keratinocyte growth factor [99, 102].

$CD4^+$ T cells differentiate into either effector T (or Th) cells or regulatory T cells. Different Th lineages such as Th1, Th2, or Th17 cells mature from common naïve precursors in secondary lymphoid tissues under the influence of specific cytokines (IL-12 for Th1; IL-4 for Th2; IL-6 and TGF-β in mice or IL-6 and IL-1 β in humans for Th17) (Figure 10.2). These cytokines are produced in response to pathogen encounter (Th1, viruses and intracellular bacteria; Th2, helminthes; Th17, intra- and extracellular bacteria and yeast) or unbalanced immune responses in the course of allergies (Th2), IBD or auto-immune diseases (both Th1 and Th17). Th cells activate innate immune cells and provide help for B cells by membrane-bound molecules and the production of cytokines such as IFNγ (Th1), IL-4/5/13 (Th2) and IL-17A/17F/21/22 (Th17) [103]. Th cell priming must be tightly regulated because uncontrolled activation leads to loss of tolerance against self and environmental antigens such as the gut microbiota. This regulation is accomplished by counter-regulatory functions of different Th cell subsets through cross-inhibition, and maybe more importantly, by the function of regulatory T cells. The latter comprise natural Tregs (nTregs) that express the transcription factor forkhead box P3 (FoxP3) which are formed in the thymus and react to self-antigens as well as inducible regulatory T cells that develop in the periphery and are specific to foreign antigens. Inducible regulatory T cells are further subdivided into so-called inducible Tregs (iTregs) that acquire FoxP3 expression in the periphery, and other FoxP3-neagtive regulatory T cells such as IL-10-producing Tr1 cells and TGF-β-producing Th3 cells. All subsets have been linked to the control of gut homeostasis and act via the production of TGF-β and IL-10 or cell–cell contact-dependent suppression of effector T cells [80, 104]. Additional $CD4^+$ T cell subsets have been described recently such as T follicular helper cells that help B cells in germinal centers to generate high-affinity antibodies, a function that has classically been ascribed to Th cells [105, 106]. These reports and others have led to debate on whether the paradigm of T-cell subsets is still useful and how to characterize phenotypic plasticity [103].

The intestinal environment strongly supports the development of Treg responses to antigens delivered by the mucosal route (Figure 10.1). Indeed, isolated LP T cells do not react to commensals. However, this tolerance is disturbed during IBD, where microbiota-specific effector T-cell responses are detectable [107]. Tolerance induction is not only confined to the local environment but also occurs systemically, as oral antigen challenge prevents effector T-cell responses to subsequent systemic immunizations with the same, but not unrelated, antigens. This

phenomenon has been termed 'oral tolerance' and has now been linked to the induction of antigen-specific Tregs [108]. Microbiota-derived signals are suggested to support T-cell homeostasis as they stimulate DCs, Mϕ, IECs, and stromal cells to produce factors such as TGF-β, IL-10, RA, and TSLP that drive the maturation of T cells with regulatory functions [80]. Interestingly, in mice, although not in humans, the pro-inflammatory effector Th17 and the immune suppressive iTregs are linked by their mutual requirement for TGF-β. However, while RA promotes intestinal induction of iTregs, IL-6 drives $CD4^+$ T-cell differentiation to Th17. At the same time, IL-6 inhibits conversion of $CD4^+$ cells to Tregs and RA inhibits the IL-6-dependent Th17 differentiation [80]. Thus, IL-6 may act as a cytokine switch that can tip this physiological inflammation over to pathological inflammation in response to microbial colonization or invasion of the mucosa when IL-6 is strongly up-regulated [95].

Importantly, experiments with germ-free and conventionalized mice revealed that the microbiota induces a constitutive development of effector T cells, in particular Th17 cells, in the intestine. An increase in Th17 levels in the small intestine was correlated with the presence of members of the cytophaga-flavobacterium-bacteroides (CFB) bacteria while selective antibiotic treatment inhibited Th17 differentiation [109]. ATP and bacterial DNA are natural adjuvants that promote homeostatic Th17 development and are suggested to play a major role in intestinal homeostasis by establishing a proper Th17/Treg balance. Higher levels of ATP in the intestinal lumen of conventionally-raised mice as compared to germ-free mice was shown to stimulate a specific subset of LP DCs to produce IL-6 and TGF-β and to promote Th17 priming [110]. Similarly, signaling of gut flora DNA through TLR9 is crucial for homeostatic Th1 and Th17 development and to limit Treg conversion [111]. Whether microbiota-induced effector T cells are microbiota specific or only result from bystander activation through the stimulation of the innate immune system remains obscure, as does their functional role. These cells may contribute to the removal of translocated commensals from the LP by antigen-specific mechanisms. Alternatively or in addition, they may help to combat invading pathogens through the presence of cross-reacting effector T cells or by non-specific bystander activation of the innate immune system. Notably, the equilibrium of effector T cells and Tregs induced by TLR9 engagement favors protective responses to oral infection and improves the outcome of vaccination [111].

10.3 Sensing, Signaling and Responding to the Microbiota to Establish a Symbiotic Relationship and Gut Homeostasis

Links between the microbial composition and health and disease have been firmly established, but the molecular mechanisms that underlie the symbiotic and pathological conditions are less well understood. Relatively recent advances in our understanding of the molecular cross talk between the gut microbiota and the host have uncovered an array of sensing capabilities, especially on the host side. It is

clear, however, that we are just starting to scratch the surface of the complex network that enables the normally peaceful intestinal habitation. On the side of the host, microbial sensing elicits changes in signaling cascades that lead to a large number of diverse outcomes that function to either establish tolerance or initiate innate and acquired immune defenses to manage a perceived threat. How intestinal microbes sense and respond to the host immune response has not received much attention but mechanisms used by pathogenic bacteria are starting to emerge.

10.3.1
Controlling Pro-inflammatory Antigens

Intestinal inflammation has dramatic effects on the density and community composition of the gut microbiota and signals a pathological condition that needs to be avoided in order to maintain a stable, healthy and symbiotic relationship. Both commensal microbes and the host contribute to the maintenance of this peaceful state by modifying lipid A, the lipid moiety of lipopolysaccharide (LPS), classically known as endotoxin, from the outer membrane of Gram-negative bacteria. Endotoxin exerts its pro-inflammatory and toxic effect by activating TLR4 and can lead to endotoxic shock and death of the host during systemic infections. Thus, while members of the *Enteroaceriaceae* family, which includes many pathogenic species, contain hexacylated lipidA with strong endotoxin activity, the lipid A of the highly abundant intestinal commensals of the phylum *Bacteroidetes* is pentacylated and exhibits non-agonistic or even antagonistic properties [112]. Since systemic circulation of endotoxin can lead to massive inflammation and shock, some Gram-negative pathogens have, however, evolved to restrict the endotoxic activity of their LPS. One striking example of this adaptation is *Yersinia pestis*, which when grown at the temperature of its flea host (25 °C) produces hexacylated LPS but tetraacyl LPS when grown at 37 °C, the normal temperature of its mammalian host, allowing it to reach high levels of bacteremia [113]. Expressing lipid A with six or more acyl chains can, however, protect bacteria from antimicrobial molecules in musosal secretions and can therefore be advantageous to pathogenic bacteria [112].

Although much of the LPS from a healthy intestinal microbiota has limited inflammatory potential, the host plays an active part in detoxifying lipid A and restricting its inflammatory activity through deacetylation and dephosphorylation (Figure 10.3(1) and 10.3(2)). One key host enzyme for LPS detoxification is acyloxyacyl hydrolase (AOAH), a lipase of Mϕs, DCs, PMNs and renal cortical epithelial cells that renders LPS non-stimulatory by removing secondary fatty acyl chains from its lipid A moiety [114]. AOAH thereby dampens LPS-induced cytokine and chemokine responses as well as B-cell proliferation and polyclonal antibody production after Gram-negative bacterial challenge [115, 116]. This dampening presumably limits the self-damage of the inflammatory response during recovery from infectious diseases. Indeed, in the urinary tract of mice where AOAH is produced by cortical renal tubule cells and is secreted into the urine, AOAH-mediated deacetylation of lipid A was shown to limit the inflammatory response

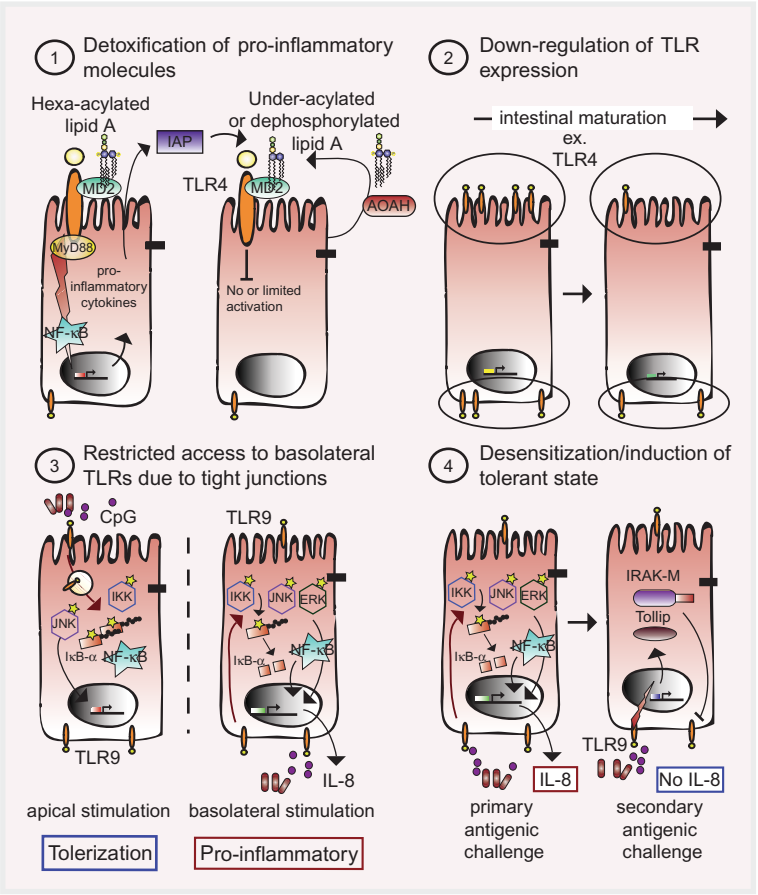


Figure 10.3 Control of inflammation at the intestinal epithelium. Various strategies are used by the host, as well as by the microbiota, to limit pro-inflammatory signaling of intestinal epithelial cells via TLRs.

to Gram-negative bacteria [117]. Infection with Gram-negative bacteria also induces an LPS-tolerant state, particularly in Mϕs and PMNs, that makes the host less responsive to subsequent infection. Interestingly, AOAH activity also restores effective host defenses after Gram-negative bacterial infections by mediating recovery from this state. AOAH-deficient mice thus display prolonged LPS tolerance after LPS challenge and become highly susceptible to a secondary peritoneal challenge with virulent *E. coli* [118]. Another host-derived LPS detoxification mechanism involves the dephosphorylation of LPS by intestinal alkaline phosphatase (IAP). In zebrafish, IAP promotes gut homeostasis and tolerance to the resident microbiota by preventing hypersensitivity to microbiota-derived LPS and intestinal inflammation [119]. IAP expression is dependent on innate TLR signaling and is localized to the apical surface of IECs, enabling detoxification of luminal LPS,

while maintaining the pro-inflammatory state of LPS in the basolateral region. In mice, IAP deficiency leads to enhanced bacterial translocation across the gut mucosal barrier [120].

10.3.2 Sensing the Presence of Microbes from Afar

As discussed previously, polarized epithelial cells are the sentinels of the mucosa, the first cellular line of defense, and it is becoming evident that these cells play a critical role in orchestrating the innate and acquired immune response. IECs have the ability to sense and distinguish between antigens located either near the apical surface, near the basolateral surface or intracellularly. In addition, IECs may be able to sense microbial factors or products that have diffused through the mucus layer and are no longer in close proximity to the originating microbe. Although the ability of bacterial products to diffuse through the mucus layer *in vivo* is largely unknown, bacterial products are more likely than whole bacteria to reach the underlying epithelium. In particular, recognition of secreted factors suggests that the epithelium may be able to sense factors meant for diffusion into the environment. This then could provide IECs with information on the location, proximity and possibly the composition of the colonizing microbes and enable them to respond more appropriately to the level of danger represented by the signal.

An emerging concept is the ability of IECs to sense, or perhaps better put 'to listen in', to the molecular cross talk between bacteria by hijacking the secreted quorum sensing molecules that are involved in establishing the social structure of bacteria and biofilm formation [121]. IECs can sense the quorum sensing pentapeptide, competence and sporulation factor (CSF) of the probiotic bacterium *Bacillus subtilis* by transporting this bacterial communication molecule through an organic cation/carnitine transporter (OCTN)-2, located on the apical brush border. Internalized CSF protects the cells against oxidant-mediated tissue damage and loss of barrier function by inducing a cytoprotective heat shock protein and activating the important survival pathways of p38 mitogen-activated protein kinase (MAPK) and protein kinase B (Akt) [122]. Notably, functional variants of the OCTN cation transporter genes are associated with CD [123]. However, quorum sensing molecules do not necessarily have to trigger cytoprotective mechanisms but can also trigger adverse effects.

Another apical membrane peptide transporter involved in sensing the bacterial community in the luminal space is the human peptide transporter 1 (*hPepT1*). HPepT1 can transport a variety of peptides as well as the bacterially-derived PMN chemotactic peptide, fMLP and muramyl dipeptide (MDP), a break-down product of the Gram-positive bacterial cell wall [124, 125]. PepT1-mediated transport of fMLP in the small intestine leads to PMN influx, the hallmark of inflammation [126]. At the same time, MDP, after uptake by hPepT1, can bind and activate NOD2, an intracellular pattern-recognition receptor, to induce expression of pro-inflammatory genes [124]. HPepT1 is most highly expressed in the jejunum and to a lesser extent in the ileum, and exhibits little or no expression in the colon.

Inflammation, however, induces expression of hPepT1 in the colon, suggesting a role for this transporter in innate host defense under both steady-state and pathological conditions [127].

10.3.3 Sensing the Presence of Microbes at the Cell Surface

The principal innate immune receptors of the host are the germ-line encoded pattern-recognition molecules (PRMs) that recognize conserved MAMPs. The vast majority of MAMPs are structural motifs that are unique to microbes of both commensal and pathogenic origin. The two major families of PRMs are restricted to distinct cellular compartments; the TLR family are membrane proteins and localize to plasma or vacuolar membranes, while NOD-like receptor (NLR) family members reside in the host cell cytosol. PRM signaling in the IECs controls the induction of innate defenses such as antimicrobial and cytokine production for the recruitment and activation of immune cells [128, 129]. In addition, MAMP-PRM recognition is central to the integration of antigen signals in antigen-presenting cells such as DCs and Mϕs as well as in the initiation of T- and B-cell immune responses. Regulatory mechanisms that control TLR-mediated signaling include the level of TLR expression, the expression of co-receptors, subcellular distribution of the TLR (i.e. apical, basolateral or endosomal), the frequency of receptor stimulation as stimulation can lead to tolerance and the combination of PRMs engaged, due to synergy of PRM-mediated signaling as well as the surrounding immunological environment, which can hinder or enhance signaling. MAMP-TLR recognition can lead to the activation of the nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) pathway, the MAPK pathways, or to pathways of the interferon regulatory factor (IRF) family. Two major pathways activated by TLR sensing of its cognate antigen are the pro-inflammatory as well as pro-apoptotic signaling pathways [130]. In the intestine, however, TLR-sensing of the microbiota by IECs and immune cells is not only important for coordinating the defense pathways that protect the host from threatening microbes, but it is also critical for maintaining intestinal homeostasis and protecting the intestinal epithelium from injury. Animals deficient in TLRs or TLR signaling are more prone to develop experimental colitis [131] or in the case of the TLR5 knock-out mouse, develop spontaneous colitis [132]. $TLR5^{-/-}$ mice are unable to sense bacterial flagellin and show an increased bacterial burden and a marked increase in hematopoietic-derived pro-inflammatory cytokine expression in the colon, suggesting that the TLR5 defect disrupts intestinal homeostasis and leads to increased microbial sensing through other TLRs. Indeed, the spontaneous colitis of $TLR5^{-/-}$ mice could be rescued by additional deletion of the LPS sensor TLR4 [132]. TLRs also protect against inflammation induced by oral administration of the epithelium-injuring agent dextran sodium sulfate (DSS), as mice deficient in either TLR2, TLR4 or MyD88, a major TLR adaptor molecule necessary for TLR signaling, were shown to be highly susceptible to DSS-induced colitis [131]. Similarly, activation of TLR3 by the receptor agonist poly(I:C) protects mice against DSS-

induced colitis [133]. The susceptibility of MyD88-deficient mice to epithelial injury was linked to dysregulated control of epithelial proliferation, reduced expression of cytoprotective heat-shock proteins in colonic epithelial cells, as well as low basal levels and lack of inducibility of cytokines that protect epithelial cells from injury by stimulating epithelial repair responses [131]. These studies reveal a microbiota-dependent role for TLRs in mediating steady-state intestinal homeostasis as well as a protective role upon colonic injury. In addition, loss of MyD88 protects mice from spontaneous colitis induced by a genetic deficiency in the anti-inflammatory cytokine IL-10 [134]. These findings suggest that while TLR signaling normally serves to protect the intestine from injury-inducing challenges, it is also a major contributor to intestinal inflammation in response to immune dysregulation. TLRs differ in their expression and sub-localization patterns and their responsiveness to their cognate antigen. TLR5 is localized on the basolateral side of polarized model IECs while TLRs 2 and 4 are predominantly apical and TLR 9 is detected on both sites. Notably, pro-inflammatory cytokine expression is elicited by only basoliteral stimulation of TLRs 3, 5 and 9 and apical stimulation of TLR 2 [135, 136]. Intestinal Mϕs and DCs can also become 'desensitized' to microbial MAMPs and display strategic expression of TLRs that generally favors an anti-inflammatory environment [46]. Tissue distribution of TLRs can also change during development, antigenic challenge and disease. For example, during postnatal development, colonization of the gastrointestinal tract leads to a down-regulation of the LPS-receptor TLR4 as well as reduced ability of TLR4 to induce inflammation, leading to an LPS-tolerant state [51] (Figure 10.3[2]). Colonic IECs constitutively express TLR3 and TLR5, while TLR2 and TLR4 are only barely detectable. In patients with IBD, TLR5 and TLR2 expression remains unchanged except for some up-regulation of TLR2 in inflammatory cells in the LP while TLR4 is highly induced in IECs in the colon and terminal ileum [137].

10.3.4
Sensing the Presence of Microbes Intracellularly

In addition to sensing microbial products from the outside and within vacuoles by TLR-mediated signaling, cytosolic surveillance of microbial-derived molecules is accomplished by a family of around 20 NLRs as well as other cytosolic receptors that recognize nucleic acids [135]. Members of NLR family include NOD1 and NOD2, which recognize peptidoglycan fragments from the bacterial cell wall, the flagellin sensor IPAF, NALP3 which responds to bacterial mRNA and endogenous danger signals such as extracellular ATP and uric acid crystals, as well as the recently discovered dsDNA sensor AIM2 [138, 139]. Three major signaling pathways are targeted by this cytosolic surveillance system: NF-κB-mediated inflammation and antimicrobial production, inflammasome-mediated inflammation and cell death, and the type 1 IFN pathway [139]. There is limited information on the role, if any, of most of these cytosolic receptors in the gut but it is clear that both NOD1 and NOD2 are important in maintaining gut homeostasis and in host defense against pathogenic bacteria at mucosal surfaces. NOD1 is ubiquitously

expressed and recognizes peptidoglycan molecules containing the amino acid meso-diaminopimelic acid, a peptidoglycan component mostly restricted to Gram-negative bacteria, while NOD2 is activated by MDP which is a component of the peptidoglycan of both Gram-positive and -negative bacteria and its expression is restricted to monocytes, Mϕs, DCs and is highly expressed in intestinal Paneth cells [140, 141]. Mice deficient in NOD1 are unable to properly control colonization of the stomach by *Helicobacter pylori*, while mice lacking NOD2 are more susceptible to oral, but not intraperitoneal, infection by *L. monocytogenes* [29, 142]. The susceptibility of $NOD2^{-/-}$ mice to *L. monocytogenes* intestinal infection was traced to a lack of NOD2-mediated α-defensin expression in Paneth cells. The importance of NOD2 in intestinal homeostasis and protection from inflammation is also highlighted by the findings that mutations in the NOD2 gene that reduce recognition of MDP are associated with severe cases of familial CD [143, 144]. Although $NOD2^{-/-}$ mice do not suffer from spontaneous inflammation, transgenic mice expressing a truncated NOD2 protein analogous to the human 3020insC allele associated with CD, were more susceptible to chemical-induced colonic inflammation [29, 145].

NODs and TLRs are involved in much signaling cross talk and their activation can lead to synergistic as well as complimentary outcomes. One example is the differential regulation of antimicrobial peptides in Paneth cells as expression of cryptdins is NOD2 dependent while RegIIIγ expression is MyD88/TLR4 dependent [29, 36, 146]. In addition, just as mice lacking NOD2 are more susceptible to intestinal infection with *L. monocytogenes*, mice that fail to express RegIIIγ due to the deletion of MyD88 were impaired in their ability to kill *L. monocytogenes* in the distal small intestine. The antimicrobial-mediated protection from enteropathogens is therefore achieved through the coordinated activity of both surface and intracellular PRRs. NLRs and TLRs also cooperate to coordinate the inflammatory response. For example, activation of TLRs or NODs leads to the production and accumulation of an inactive pro-form of the potent pro-inflammatory cytokine IL-1β. This primes the cell to respond to subsequent cytosolic recognition of microbial or host-derived danger signals by NLRs that activate the inflammasome and lead to the activation of the cysteine protease caspase-1, which processes pro-IL-1β to produce its mature and active form and leads to its release from the cell [147]. In addition, NOD1 and 2 can act in synergy with TLRs to enhance immune responses of antigen-presenting cells. One example is the synergistic effect of NOD1 and 2 agonists with LPS to boost production of antigen-presenting cells, pro-inflammatory cytokines such as TNF and IL-6 in human monocytes and DCs [148].

10.3.5 The Master Regulator of Intestinal Inflammation and Homeostasis: The NF-κB Signaling Pathway

NF-κB is an evolutionarily conserved transcription factor that is central to immune regulation. The term NF-κB is used collectively to describe the DNA-binding

transcription factors of the Rel family. NF-κB regulates both innate and adaptive immune responses and mediates inflammation in the gut by increasing expression of a diverse set of factors including pro-inflammatory cytokines, chemokines, cell surface receptors, anti-apoptotic factors and adhesion molecules. This master regulator of inflammation is situated at a common node in the signaling cascades for the NOD proteins and many TLRs, and not surprisingly is regulated at multiple levels. NF-κB acts as a dimer and is retained in an inactive state in the cytoplasm through association with its inhibitor IκB-α. Upon immune stimulation, IκB-α is phosphorylated by the IκB kinase (IKK) complex, which targets IκB-α for ubiquitination by the ubiquitin ligase E3-$SCF^{\beta\text{-}TrCP}$ and subsequent degradation by the proteasome. Dissociation and degradation of the inhibitory subunit unmasks the nuclear import sequence of NF-κB and allows it to translocate into the nucleus where it binds specific NF-κB enhancer sequences and together with other factors regulates expression of pro-inflammatory cytokines and innate defense mediators. In the nucleus, IκB-α dissociates NF-κB from DNA and transports it back to the cytoplasm via active CRM1-mediated nuclear export. NF-κB-mediated production of pro-inflammatory cytokines is an important signaling cascade activated in phagocytes, especially macrophages, and serves to defend the host against intestinal pathogens. It is becoming clear, however, that in addition to its role in activating the inflammatory response, NF-κB signaling, and particularly that in gut epithelial cells, is also required to maintain intestinal homeostasis [39, 149].

In IECs, NF-κB activation is regulated by domain-specific engagement of TLR signaling. Indeed, directional signaling of TLRs is emerged as an important mechanism that provides critical information on the danger level of the stimulant. A striking example of how domain-specific recognition of a TLR ligand leads to differences in the response pattern crucial to the maintenance of homeostasis has been elucidated for TLR9 [150]. TLR9 recognizes bacterial non-methylated CpG and can be engaged from both the apical and basolateral side of polarized human IECs. It was shown that apical TLR9 stimulation of polarized IECs leads to Jun-N-terminal kinase (JNK) and IKK kinase activation but surprisingly poly-ubiquitinated IκB-α accumulated and NF-κB remained in the cytosol. Conversely, basolateral stimulation activated the JNK and Erk pathways, lead to the degradation of IκB-α, activation of NF-κB and a robust inflammatory response as measured by production of the PMN chemokine CXCL8 (Figure 10.3(3)). Furthermore, apical TLR9 engagement led to a more tolerant cellular state whereby CXCL8 production induced by basolateral TLR9 stimulation was suppressed in a time-dependent manner (Figure 10.3(4)). Interestingly, under conditions where basolateral TLR9 signaling induces inflammation, an apparently separate mechanism triggers a tolerogenic state in IECs to again limit the inflammatory response. Instead of accumulating poly-ubiquitinated IκB-α, which presumably interferes with nuclear translocation of NF-κB, basolaterally pre-stimulated IECs expressed inhibitory molecules to TLR signaling, such as IRAK-M and Tollip, in response to a second antigenic challenge. Together these findings reveal that while basolateral engagement of TLR9 of IECs leads to an inflammatory response, apical TLR9 signaling protects from inflammation. Thus, under steady-state conditions where microbial

products are predominantly located on the luminal side of the IEC monolayer, TLR9 sensing and differential regulation of the NF-κB pathway contributes to a tolerogenic state and maintains gut homeostasis.

The protective role of the NF-κB pathway in IECs for intestinal homeostasis and epithelial integrity has been dissected by IEC-specific ablation of single and multiple components of the IKK complex required for the activation of NF-κB [39, 149]. IKK is composed of the regulatory subunit NEMO (also called IKKγ) and two kinases (IKKα and IKKβ), of which IKKβ is the major kinase for canonical NF-κB activation. IEC-specific IKKβ knock-out mice display an increased susceptibility to infection as well as intestinal injury, while double knock-outs of IKKα and IKKβ, or ablation of the regulatory subunit NEMO, leads to translocation of commensal bacteria into the mucosa, dysregulated expression of pro-inflammatory cytokines by DCs and spontaneous intestinal inflammation reminiscent of IBD. Signaling through either IKKα or IKKβ by IECs is therefore necessary to maintain the integrity of the mucosal barrier under steady-state conditions while both are required to protect the mucosa from pathogenic or chemical assault. The inflammatory phenotype could be rescued by deleting MyD88, revealing the necessity for TLR activation in the disease progression. In addition, loss of NF-κB signaling by IKKγ deletion also led to apoptosis of colonic ECs, increased sensitivity of ECs to TNF-induced apoptosis and impaired expression of antimicrobial factors revealing a critical role for NF-κB in maintaining a 'pro-life' program in the IECs that protects against TNF-induced apoptosis [39, 149].

Given the central regulatory role that NF-κB plays in the inflammatory response, apoptosis and mucosal homeostasis in the intestine, it is not surprising that it has emerged as a major target for manipulation by both commensal and pathogenic bacteria. The importance of NF-κB regulation is highlighted by the many ways in which microbes have found to manipulate its regulation. It has been shown that gut commensals, particularly the prevalent colonic anaerobe *B. thetaiotoamicron*, can attenuate the inflammatory response to virulent *S. enteritidis* and bacterial flagellin by promoting the nucleo-cytoplasmic shuttling of NF-κB. *B. thetaiotoamicron* does this by up-regulating the orphan nuclear receptor the peroxisome proliferator-activated receptor gamma (PPARγ), which negatively regulates NF-κB activity by enhancing the nuclear export of the RelA subunit of NF-κB through a novel Crm-1-independent pathway [151]. In respiratory ECs, it has been shown that *Bordetella bronchiseptica* suppresses TLR-mediated activation of NF-κB and the subsequent up-regulation of β-defensin tracheal antimicrobial peptide (TAP) and can block TNF-induced NF-κB activation by inhibiting cytoplasmic to nuclear shuttling of the NF-κB p65 subunit, resulting in apoptotic death of the EC [152, 153]. More commonly, gut commensals and pathogenic bacteria prevent or attenuate inflammation by preventing activation of NF-κB rather than by interfering with its nuclear shuttling. For example, pre-incubation of IECs with *Lactobacillus casei* was shown to inhibit transcription of a number of pro-inflammatory effectors and adherence molecules induced by a subsequent infection with *S. flexneri*. This inhibitory effect was mediated at least partially by *L. casei*-dependent stabilization of phosphorylated IκB-α, possibly due to a down-regulation of several

components of the proteasome as well as ROC-1, a component of the ubiquitin ligase E3-SCF$^{\beta\text{-TrCP}}$ [154]. Like probiotic bacteria, live non-virulent *Salmonella* can abrogate IκB-α degradation by blocking ubiqutination of phosphorylated IκB-α and can prevent activation of NF-κB by pro-inflammatory cytokines such as TNF [155]. Notably, interference with the eukaryotic ubiquitin degradation pathway (UPS) by bacterial type III or type IV secretion effectors has emerged as a general tactic by which pathogens subvert host functions [156]. Intriguingly, it was recently shown that the gut microbiota can also stimulate host cells to inhibit NF-κB activation by inducing the production of reactive oxygen species that target the degradative machinery of the host cell. In this elegant study, the production of reactive oxygen species prevented the formation of a thioester bond between the ubiquitin-like protein NEDD8 and the NEDD8-conjugating E2 enzyme Ubc12, leading to a lack of neddylated Cullin-1 which serves as a scaffolding protein for the ubiquitin ligase E3-SCF$^{\beta\text{-TrCP}}$ and is required for its ligase activity. The inhibition of E3-SCF$^{\beta\text{-TrCP}}$ blocks IκB-α degradation and hence activation of NF-κB [151, 157]. The *de novo* and transient ROS generating activity and downstream inhibition of Ubc12 neddylation by enteric commensal bacteria could be recapitulated *in vitro* and *ex vivo* by the addition of the bacterial fermentation end-product butyrate and other short chain fatty acids when the environment was at a slightly acidic pH, similar to that in the proximal ascending colon (pH 5.5 to 6.6) where abundant fermentation activity leads to a high production of organic acids [158]. These studies provide a mechanistic link to the previously observed anti-inflammatory actions of short-chain fatty acids [159, 160] that have led to their therapeutic use in dampening inflammation in inflammatory bowl diseases [161].

10.4 Conclusion

We have described here numerous mechanisms present in humans and mice that permit a symbiotic relationship between the host and the microbiota while at the same time allowing the host to protect itself against pathogenic intrusion. Commensal and pathogenic bacteria share the same molecular motifs that are recognized by the host's innate immune surveillance system. The strict compartmentalization of commensal bacteria within the intestinal lumen by the pre-epithelial barrier is probably the key to understanding why these bacteria do not trigger overactive inflammatory responses [162]. However, the gut barrier exhibits a certain degree of leakiness and commensals can reach the epithelium and even cross this barrier to access the underlying LP. Microbiota-derived signals drive the development of the epithelium but they are also important for its metabolic and immunological maturation both systemically and in the intestine. This suggests that this 'leakiness' is not accidental but rather controlled and, thus, advantageous for the host. The intestinal immune system is therefore endowed with a substantial capacity to mount tolerizing immune responses in order to sustain homeostasis with the minor fraction of commensals that cross

the epithelial barrier. Except for some opportunistic pathogens that are part of the indigenous gut flora, professional pathogens generally cannot compete with the microbiota within the lumen but instead have developed mechanisms to overcome the intestinal barriers in order to find different environmental niches in which they can thrive [1]. The incoming signals delivered by pathogens probably differ in quality from those of commensals (e.g. the presence of toxins and other bacterial effectors as well as intracellular or intracytosolic localization) and in quantity (e.g. the enhanced survival near the epithelium or within the LP results in higher concentrations of pro-inflammatory MAMPs). Therefore, the host immune system recognizes 'danger' and a switch from a tolerizing to a pro-inflammatory effector immune response follows [1].

Tolerance to the microbiota can be disrupted as a result of changes in the microbiota composition, disrupted barrier functions, and/or imbalance of the immune regulation. The understanding of the complex regulatory networks of gut homeostasis is not predictable from simple models but requires fundamental analysis of the multi-factorial signaling networks at a temporal and spatial resolution. For example, disruption of TLR signaling can lead to increased or decreased intestinal inflammation depending on the experimental model. Colitis models that mainly target epithelial integrity, such as DSS challenge, show enhanced pathology in several knock-out mice with defects in TLRs or its downstream targets [131]. However, IL-10 knock-out mice that exhibit a dysregulation of immune cells within the LP and show spontaneous colitis are protected from colitis when TLR signaling is disrupted [134]. Clearly, the location at which TLR ligands are recognized is the key to understanding whether these molecules promote or ameliorate the disease. Cell-specific knock-outs will therefore be needed to address the contribution of specific molecules to the regulation of gut homeostasis.

During the last two decades a large number of animal models using genetically-deficient mice have shed light on how tolerance to the microbiota is achieved [163]. In addition, the list of gene defects that are linked to IBD in humans is constantly growing [164]. In contrast, the environmental factors, such as specific commensal strains or nutrition, that lead to the loss of tolerance to gut flora in IBD patients are still poorly defined [165]. A better understanding of the GI barrier and underlying immune surveillance on the one hand, and the diversity, biogeography, and metabolic and immunological characteristics of the microbiota and their change in the course of IBD on the other hand, will hopefully lead to optimized treatment options.

Another important area that will likely benefit from a more complete understanding of the cellular and molecular details that lead to gut homeostasis involves treatments against GI pathogens. Unfortunately, the study of gut pathogens *in vivo* is often hampered by the lack of suitable animal models, which, however, is a prerequisite for understanding the virulence strategy of the pathogen and the immune response of the host. Importantly, vaccines are still the most promising tool for the prevention of infectious diseases [166]. However, there are often insufficient vaccine candidates for GI pathogens. This may be explained by the general tolerizing immune responses that are mounted in the gut if antigens are presented

in the non- or low-inflammatory context in which vaccines generally need to be administered in order to be tolerated. Whereas the induction of tolerance by mucosal-delivered antigens can be used to treat allergies and possibly autoimmune diseases, this strategy is contradictory for a successful vaccine against intestinal pathogens. Strategies that overcome this problem therefore have to be developed and could possibly include new delivery systems with adjuvant properties [167], mixed systemic and mucosal prime-boost regimes [168], or specific targeting of T cell-independent IgA responses [169].

Abbreviations

Ang, angiogenin; AOAH, acyloxyacyl hydrolase; APRIL, a proliferation-inducing ligand; CCL, CC chemokine ligand; CD40L, CD40 ligand; CSF, competence and sporulation factor; CSR, class switch reaction; CD, Crohn's disease; DC, dendritic cell; DSS, dextran sodium sulfate; EC, epithelial cell; EHEC, enterohemorrhagic *E. coli*; FcRn, neonatal Fc receptor; fMLP, formyl-met-leu-phe; FoxP3, forkhead box P3; GALT, gut-associated lymphoid tissue; GI, gastrointestinal; *hPepT1,* human peptide transporter 1; IAP, intestinal alkaline phosphatase; IBD, inflammatory bowl disease; IEC, intestinal epithelial cell; IEL, intraepithelial lymphocyte; IFN, interferon; IKK, IκB kinase; IL, interleukin; ILF, isolated lymphoid follicle; iNOS, inducible-nitric-oxide-synthase; iTregs, inducible regulatory T cell; JNK, Jun-N-terminal kinase; LPS, lipopolysaccharide; LN, lymph node; LP, lamina propria; LPS, lipopolysaccharide; M cell, microfold cell; Mϕ, macrophage; MAMP, microbe-associated molecular pattern; MAPK, mitogen-activated protein kinase; MDP, muramyl dipeptide; MLN, mesenteric lymph node; NF-κB, nuclear factor kappa-light-chain-enhancer of activated B cells; NK, natural killer; NLR, NOD-like receptor; NOD, nucleotide-binding oligomerization domain-containing; nTregs, natural regulatory T cell; OCTN, cation/carnitine transporter; pIgR, polymeric immunoglobulin receptor; PMN, polymorphonuclear cell; PP, Peyer's Patch; PPARγ, peroxisome proliferator-activated receptor gamma; PRM, pattern-recognition molecules; RA, retinoic acid; ROS, reactive oxygen species; SIgA, secretory IgA; TAP, tracheal antimicrobial peptide; TCR, T cell receptor; TFF, Trefoil factor; TGF-β, transforming growth factor beta; Th, T helper; TLR, Toll-like receptor; TNF, tumor necrosis factor; Treg, regulatory T cell; TSLP, thymic stromal lymphopoietin; UPS, ubiquitin degradation pathway.

References

1 Sansonetti, P.J. (2004) *Nat. Rev. Immunol.*, **4**, 953–964.
2 Brandtzaeg, P., Kiyono, H., Pabst, R., and Russell, M.W. (2008) *Mucosal. Immunol.*, **1**, 31–37.
3 Ley, R.E., Peterson, D.A., and Gordon, J.I. (2006) *Cell*, **124**, 837–848.
4 Ando, H., Abe, H., Sugimoto, N., and Tobe, T. (2007) *Microbiology*, **153**, 464–473.
5 Atuma, C., Strugala, V., Allen, A., and Holm, L. (2001) *Am. J. Physiol. Gastrointest.. Liver Physiol.*, **280**, G922–G929.

6 Specian, R.D., and Neutra, M.R. (1980) *J. Cell Biol.*, **85**, 626–640.

7 Comelli, E.M., Simmering, R., Faure, M., Donnicola, D., Mansourian, R., Rochat, F., Corthesy-Theulaz, I., and Cherbut, C. (2008) *Genomics*, **91**, 70–77.

8 Ensgraber, M., Genitsariotis, R., Storkel, S., and Loos, M. (1992) *Microb. Pathog.*, **12**, 255–266.

9 Sajjan, S.U., and Forstner, J.F. (1990) *Infect. Immun.*, **58**, 868–873.

10 Smith, C.J., Kaper, J.B., and Mack, D.R. (1995) *J. Pediatr. Gastroenterol. Nutr.*, **21**, 269–276.

11 Meyer-Hoffert, U., Hornef, M.W., Henriques-Normark, B., Axelsson, L.G., Midtvedt, T., Putsep, K., and Andersson, M. (2008) *Gut*, **57**, 764–771.

12 Phalipon, A., Cardona, A., Kraehenbuhl, J.P., Edelman, L., Sansonetti, P.J., and Corthesy, B. (2002) *Immunity*, **17**, 107–115.

13 Taupin, D., and Podolsky, D.K. (2003) *Nat. Rev. Mol. Cell Biol.*, **4**, 721–732.

14 Dignass, A., Lynch-Devaney, K., Kindon, H., Thim, L., and Podolsky, D.K. (1994) *J. Clin. Invest.*, **94**, 376–383.

15 Swidsinski, A., Weber, J., Loening-Baucke, V., Hale, L.P., and Lochs, H. (2005) *J. Clin. Microbiol.*, **43**, 3380–3389.

16 Einerhand, A.W., Renes, I.B., Makkink, M.K., van der Sluis, M., Buller, H.A., and Dekker, J. (2002) *Eur. J. Gastroenterol. Hepatol.*, **14**, 757–765.

17 van der Sluis, M., de Koning, B.A., De Bruijn, A.C., Velcich, A., Meijerink, J.P., van Goudoever, J.B., Buller, H.A., Dekker, J., Van Seuningen, I., Renes, I.B., and Einerhand, A.W. (2006) *Gastroenterology*, **131**, 117–129.

18 Velcich, A., Yang, W., Heyer, J., Fragale, A., Nicholas, C., Viani, S., Kucherlapati, R., Lipkin, M., Yang, K., and Augenlicht, L. (2002) *Science*, **295**, 1726–1729.

19 Mashimo, H., Wu, D.C., Podolsky, D.K., and Fishman, M.C. (1996) *Science*, **274**, 262–265.

20 Schneider, D.R., and Parker, C.D. (1982) *J. Infect. Dis.*, **145**, 474–482.

21 Lievin-Le Moal, V., and Servin, A.L. (2006) *Clin. Microbiol. Rev.*, **19**, 315–337.

22 Guttman, J.A., and Finlay, B.B. (2009) *Biochim. Biophys. Acta*, **1788**, 832–841.

23 Artis, D. (2008) *Nat. Rev. Immunol.*, **8**, 411–420.

24 Rescigno, M., Lopatin, U., and Chieppa, M. (2008) *Curr. Opin. Immunol.*, **20**, 669–675.

25 Cerutti, A. (2008) *Nat. Rev. Immunol.*, **8**, 421–434.

26 Saenz, S.A., Taylor, B.C., and Artis, D. (2008) *Immunol. Rev.*, **226**, 172–190.

27 Booth, C., and Potten, C.S. (2000) *J. Clin. Invest.*, **105**, 1493–1499.

28 Ortega-Cava, C.F., Ishihara, S., Rumi, M.A., Kawashima, K., Ishimura, N., Kazumori, H., Udagawa, J., Kadowaki, Y., and Kinoshita, Y. (2003) *J. Immunol.*, **170**, 3977–3985.

29 Kobayashi, K.S., Chamaillard, M., Ogura, Y., Henegariu, O., Inohara, N., Nunez, G., and Flavell, R.A. (2005) *Science*, **307**, 731–734.

30 Hermiston, M.L., and Gordon, J.I. (1995) *Science*, **270**, 1203–1207.

31 Salzman, N.H., Underwood, M.A., and Bevins, C.L. (2007) *Semin. Immunol.*, **19**, 70–83.

32 Mukherjee, S., Vaishnava, S., and Hooper, L.V. (2008) *Cell Mol. Life Sci.*, **65**, 3019–3027.

33 Hooper, L.V., Stappenbeck, T.S., Hong, C.V., and Gordon, J.I. (2003) *Nat. Immunol.*, **4**, 269–273.

34 Cash, H.L., Whitham, C.V., Behrendt, C.L., and Hooper, L.V. (2006) *Science*, **313**, 1126–1130.

35 Ayabe, T., Satchell, D.P., Wilson, C.L., Parks, W.C., Selsted, M.E., and Ouellette, A.J. (2000) *Nat. Immunol.*, **1**, 113–118.

36 Vaishnava, S., Behrendt, C.L., Ismail, A.S., Eckmann, L., and Hooper, L.V. (2008) *Proc. Natl Acad. Sci. USA*, **105**, 20858–20863.

37 Sonnenburg, J.L., Chen, C.T., and Gordon, J.I. (2006) *PLoS Biol.*, **4**, e413.

38 Sperandio, B., Regnault, B., Guo, J., Zhang, Z., Stanley, S.L., Jr., Sansonetti, P.J., and Pedron, T. (2008) *J. Exp. Med.*, **205**, 1121–1132.

39 Nenci, A., Becker, C., Wullaert, A., Gareus, R., van Loo, G., Danese, S., Huth, M., Nikolaev, A., Neufert, C., Madison, B., Gumucio, D., Neurath, M.F., and Pasparakis, M. (2007) *Nature*, **446**, 557–561.

40 Wilson, C.L., Ouellette, A.J., Satchell, D.P., Ayabe, T., Lopez-Boado, Y.S., Stratman, J.L., Hultgren, S.J., Matrisian, L.M., and Parks, W.C. (1999) *Science*, **286**, 113–117.

41 Brandl, K., Plitas, G., Mihu, C.N., Ubeda, C., Jia, T., Fleisher, M., Schnabl, B., DeMatteo, R.P., and Pamer, E.G. (2008) *Nature*, **455**, 804–807.

42 Salzman, N.H., Ghosh, D., Huttner, K.M., Paterson, Y., and Bevins, C.L. (2003) *Nature*, **422**, 522–526.

43 Wehkamp, J., Harder, J., Weichenthal, M., Schwab, M., Schaffeler, E., Schlee, M., Herrlinger, K.R., Stallmach, A., Noack, F., Fritz, P., Schroder, J.M., Bevins, C.L., Fellermann, K., and Stange, E.F. (2004) *Gut*, **53**, 1658–1664.

44 Ryu, J.H., Kim, S.H., Lee, H.Y., Bai, J.Y., Nam, Y.D., Bae, J.W., Lee, D.G., Shin, S.C., Ha, E.M., and Lee, W.J. (2008) *Science*, **319**, 777–782.

45 Ohnmacht, C., and Voehringer, D. (2009) *Blood*, **113**, 2816–2825.

46 Smythies, L.E., Sellers, M., Clements, R.H., Mosteller-Barnum, M., Meng, G., Benjamin, W.H., Orenstein, J.M., and Smith, P.D. (2005) *J. Clin. Invest.*, **115**, 66–75.

47 Smith, P.D., Smythies, L.E., Mosteller-Barnum, M., Sibley, D.A., Russell, M.W., Merger, M., Sellers, M.T., Orenstein, J.M., Shimada, T., Graham, M.F., and Kubagawa, H. (2001) *J. Immunol.*, **167**, 2651–2656.

48 Denning, T.L., Wang, Y.C., Patel, S.R., Williams, I.R., and Pulendran, B. (2007) *Nat. Immunol.*, **8**, 1086–1094.

49 Bjerke, K., Halstensen, T.S., Jahnsen, F., Pulford, K., and Brandtzaeg, P. (1993) *Gut*, **34**, 1357–1363.

50 Macpherson, A.J., and Uhr, T. (2004) *Science*, **303**, 1662–1665.

51 Lotz, M., Gutle, D., Walther, S., Menard, S., Bogdan, C., and Hornef, M.W. (2006) *J. Exp. Med.*, **203**, 973–984.

52 Pull, S.L., Doherty, J.M., Mills, J.C., Gordon, J.I., and Stappenbeck, T.S. (2005) *Proc. Natl Acad. Sci. USA*, **102**, 99–104.

53 Dunay, I.R., Damatta, R.A., Fux, B., Presti, R., Greco, S., Colonna, M., and Sibley, L.D. (2008) *Immunity*, **29**, 306–317.

54 Kramer, S., Sellge, G., Lorentz, A., Krueger, D., Schemann, M., Feilhauer, K., Gunzer, F., and Bischoff, S.C. (2008) *J. Immunol.*, **181**, 1438–1445.

55 Bischoff, S.C. (2007) *Nat. Rev. Immunol.*, **7**, 93–104.

56 Bischoff, S.C., Lorentz, A., Schwengberg, S., Weier, G., Raab, R., and Manns, M.P. (1999) *Gut*, **44**, 643–652.

57 Malaviya, R., Ikeda, T., Ross, E., and Abraham, S.N. (1996) *Nature*, **381**, 77–80.

58 Echtenacher, B., Mannel, D.N., and Hultner, L. (1996) *Nature*, **381**, 75–77.

59 Hogan, S.P., Rosenberg, H.F., Moqbel, R., Phipps, S., Foster, P.S., Lacy, P., Kay, A.B., and Rothenberg, M.E. (2008) *Clin. Exp. Allergy*, **38**, 709–750.

60 Bischoff, S.C., Wedemeyer, J., Herrmann, A., Meier, P.N., Trautwein, C., Cetin, Y., Maschek, H., Stolte, M., Gebel, M., and Manns, M.P. (1996) *Histopathology*, **28**, 1–13.

61 Kato, M., Kephart, G.M., Talley, N.J., Wagner, J.M., Sarr, M.G., Bonno, M., McGovern, T.W., and Gleich, G.J. (1998) *Anat. Rec.*, **252**, 418–425.

62 Cella, M., Fuchs, A., Vermi, W., Facchetti, F., Otero, K., Lennerz, J.K., Doherty, J.M., Mills, J.C., and Colonna, M. (2009) *Nature*, **457**, 722–725.

63 Satoh-Takayama, N., Vosshenrich, C.A., Lesjean-Pottier, S., Sawa, S., Lochner, M., Rattis, F., Mention, J.J., Thiam, K., Cerf-Bensussan, N., Mandelboim, O., Eberl, G., and Di Santo, J.P. (2008) *Immunity*, **29**, 958–970.

64 Buning, J., Hundorfean, G., Schmitz, M., Zimmer, K.P., Strobel, S., Gebert, A., and Ludwig, D. (2006) *FASEB J.*, **20**, 359–361.

65 Husby, S., Foged, N., Host, A., and Svehag, S.E. (1987) *Gut*, **28**, 1062–1072.

66 Xu, X.L., Lee, R.T., Fang, H.M., Wang, Y.M., Li, R., Zou, H., Zhu, Y., and Wang, Y. (2008) *Cell Host Microbe*, **4**, 28–39.

67 Niess, J.H. (2008) *World J. Gastroenterol.*, **14**, 5138–5148.

68 Jang, M.H., Kweon, M.N., Iwatani, K., Yamamoto, M., Terahara, K., Sasakawa, C., Suzuki, T., Nochi, T., Yokota, Y., Rennert, P.D., Hiroi, T., Tamagawa, H.,

Iijima, H., Kunisawa, J., Yuki, Y., and Kiyono, H. (2004) *Proc. Natl Acad. Sci. USA*, **101**, 6110–6115.
69 Mach, J., Hshieh, T., Hsieh, D., Grubbs, N., and Chervonsky, A. (2005) *Immunol. Rev.*, **206**, 177–189.
70 Yoshida, M., Kobayashi, K., Kuo, T.T., Bry, L., Glickman, J.N., Claypool, S.M., Kaser, A., Nagaishi, T., Higgins, D.E., Mizoguchi, E., Wakatsuki, Y., Roopenian, D.C., Mizoguchi, A., Lencer, W.I., and Blumberg, R.S. (2006) *J. Clin. Invest.*, **116**, 2142–2151.
71 Yoshida, M., Claypool, S.M., Wagner, J.S., Mizoguchi, E., Mizoguchi, A., Roopenian, D.C., Lencer, W.I., and Blumberg, R.S. (2004) *Immunity*, **20**, 769–783.
72 Rescigno, M., Rotta, G., Valzasina, B., and Ricciardi-Castagnoli, P. (2001) *Immunobiology*, **204**, 572–581.
73 Chieppa, M., Rescigno, M., Huang, A.Y., and Germain, R.N. (2006) *J. Exp. Med.*, **203**, 2841–2852.
74 Niess, J.H., Brand, S., Gu, X., Landsman, L., Jung, S., McCormick, B.A., Vyas, J.M., Boes, M., Ploegh, H.L., Fox, J.G., Littman, D.R., and Reinecker, H.C. (2005) *Science*, **307**, 254–258.
75 Coombes, J.L., and Powrie, F. (2008) *Nat. Rev. Immunol.*, **8**, 435–446.
76 Iwasaki, A., and Kelsall, B.L. (1999) *J. Exp. Med.*, **190**, 229–239.
77 Millard, A.L., Mertes, P.M., Ittelet, D., Villard, F., Jeannesson, P., and Bernard, J. (2002) *Clin. Exp. Immunol.*, **130**, 245–255.
78 Rimoldi, M., Chieppa, M., Salucci, V., Avogadri, F., Sonzogni, A., Sampietro, G.M., Nespoli, A., Viale, G., Allavena, P., and Rescigno, M. (2005) *Nat. Immunol.*, **6**, 507–514.
79 Hammerschmidt, S.I., Ahrendt, M., Bode, U., Wahl, B., Kremmer, E., Forster, R., and Pabst, O. (2008) *J. Exp. Med.*, **205**, 2483–2490.
80 Mucida, D., Park, Y., and Cheroutre, H. (2009) *Semin. Immunol.*, **21**, 14–21.
81 Uematsu, S., Fujimoto, K., Jang, M.H., Yang, B.G., Jung, Y.J., Nishiyama, M., Sato, S., Tsujimura, T., Yamamoto, M., Yokota, Y., Kiyono, H., Miyasaka, M., Ishii, K.J., and Akira, S. (2008) *Nat. Immunol.*, **9**, 769–776.
82 Salazar-Gonzalez, R.M., Niess, J.H., Zammit, D.J., Ravindran, R., Srinivasan, A., Maxwell, J.R., Stoklasek, T., Yadav, R., Williams, I.R., Gu, X., McCormick, B.A., Pazos, M.A., Vella, A.T., Lefrancois, L., Reinecker, H.C., and McSorley, S.J. (2006) *Immunity*, **24**, 623–632.
83 Cerutti, A., and Rescigno, M. (2008) *Immunity*, **28**, 740–750.
84 Targan, S.R., Landers, C.J., Yang, H., Lodes, M.J., Cong, Y., Papadakis, K.A., Vasiliauskas, E., Elson, C.O., and Hershberg, R.M. (2005) *Gastroenterology*, **128**, 2020–2028.
85 Corthesy, B. (2007) *J. Immunol.*, **178**, 27–32.
86 Peterson, D.A., McNulty, N.P., Guruge, J.L., and Gordon, J.I. (2007) *Cell Host Microbe*,**2**, 328–339.
87 Phalipon, A., Michetti, P., Kaufmann, M., Cavaillon, J.M., Huerre, M., Kraehenbuhl, J.P., and Sansonetti, P.J. (1994) *Ann. NY Acad. Sci.*, **730**, 356–358.
88 Jacob, C.M., Pastorino, A.C., Fahl, K., Carneiro-Sampaio, M., and Monteiro, R.C. (2008) *J. Clin. Immunol.*, **28** (Suppl .1), S56–S61.
89 Suzuki, K., Meek, B., Doi, Y., Muramatsu, M., Chiba, T., Honjo, T., and Fagarasan, S. (2004) *Proc. Natl Acad. Sci. USA*, **101**, 1981–1986.
90 Suzuki, K., and Fagarasan, S. (2008) *Trends Immunol.*, **29**, 523–531.
91 Macpherson, A.J., Gatto, D., Sainsbury, E., Harriman, G.R., Hengartner, H., and Zinkernagel, R.M. (2000) *Science*, **288**, 2222–2226.
92 Tezuka, H., Abe, Y., Iwata, M., Takeuchi, H., Ishikawa, H., Matsushita, M., Shiohara, T., Akira, S., and Ohteki, T. (2007) *Nature*, **448**, 929–933.
93 Murakami, M., Tsubata, T., Shinkura, R., Nisitani, S., Okamoto, M., Yoshioka, H., Usui, T., Miyawaki, S., and Honjo, T. (1994) *J. Exp. Med.*, **180**, 111–121.
94 Wijburg, O.L., Uren, T.K., Simpfendorfer, K., Johansen, F.E., Brandtzaeg, P., and Strugnell, R.A. (2006) *J. Exp. Med.*, **203**, 21–26.
95 Sansonetti, P.J., and Di Santo, J.P. (2007) *Immunity*, **26**, 149–161.

96 Macpherson, A.J., Geuking, M.B., and McCoy, K.D. (2005) *Immunology*, **115**, 153–162.

97 Izcue, A., Coombes, J.L., and Powrie, F. (2006) *Immunol. Rev.*, **212**, 256–271.

98 Treiner, E., and Lantz, O. (2006) *Curr. Opin. Immunol.*, **18**, 519–526.

99 Kunisawa, J., Takahashi, I., and Kiyono, H. (2007) *Immunol. Rev.*, **215**, 136–153.

100 Bauer, S., Groh, V., Wu, J., Steinle, A., Phillips, J.H., Lanier, L.L., and Spies, T. (1999) *Science*, **285**, 727–729.

101 Groh, V., Steinle, A., Bauer, S., and Spies, T. (1998) *Science*, **279**, 1737–1740.

102 Cheroutre, H. (2005) *Immunol. Rev.*, **206**, 114–131.

103 Zhou, L., Chong, M.M., and Littman, D.R. (2009) *Immunity*, **30**, 646–655.

104 Sakaguchi, S., Yamaguchi, T., Nomura, T., and Ono, M. (2008) *Cell*, **133**, 775–787.

105 Tsuji, M., Komatsu, N., Kawamoto, S., Suzuki, K., Kanagawa, O., Honjo, T., Hori, S., and Fagarasan, S. (2009) *Science*, **323**, 1488–1492.

106 Silver, J.S., and Hunter, C.A. (2008) *Immunity*, **29**, 7–9.

107 Duchmann, R., Kaiser, I., Hermann, E., Mayet, W., Ewe, K., and Meyer zum Buschenfelde, K.H. (1995) *Clin. Exp. Immunol.*, **102**, 448–455.

108 Faria, A.M., and Weiner, H.L. (2005) *Immunol. Rev.*, **206**, 232–259.

109 Ivanov, I.I., Frutos, R.L., Manel, N., Yoshinaga, K., Rifkin, D.B., Sartor, R.B., Finlay, B.B., and Littman, D.R. (2008) *Cell Host Microbe*, **4**, 337–349.

110 Atarashi, K., Nishimura, J., Shima, T., Umesaki, Y., Yamamoto, M., Onoue, M., Yagita, H., Ishii, N., Evans, R., Honda, K., and Takeda, K. (2008) *Nature*, **455**, 808–812.

111 Hall, J.A., Bouladoux, N., Sun, C.M., Wohlfert, E.A., Blank, R.B., Zhu, Q., Grigg, M.E., Berzofsky, J.A., and Belkaid, Y. (2008) *Immunity*, **29**, 637–649.

112 Munford, R.S. (2008) *Infect. Immun.*, **76**, 454–465.

113 Montminy, S.W., Khan, N., McGrath, S., Walkowicz, M.J., Sharp, F., Conlon, J.E., Fukase, K., Kusumoto, S., Sweet, C., Miyake, K., Akira, S., Cotter, R.J., Goguen, J.D., and Lien, E. (2006) *Nat. Immunol.*, **7**, 1066–1073.

114 Munford, R.S., and Hall, C.L. (1986) *Science*, **234**, 203–205.

115 Lu, M., Zhang, M., Takashima, A., Weiss, J., Apicella, M.A., Li, X.H., Yuan, D., and Munford, R.S. (2005) *Nat. Immunol.*, **6**, 989–994.

116 Kitchens, R.L., Ulevitch, R.J., and Munford, R.S. (1992) *J. Exp. Med.*, **176**, 485–494.

117 Feulner, J.A., Lu, M., Shelton, J.M., Zhang, M., Richardson, J.A., and Munford, R.S. (2004) *Infect. Immun.*, **72**, 3171–3178.

118 Lu, M., Varley, A.W., Ohta, S., Hardwick, J., and Munford, R.S. (2008) *Cell Host Microbe*, **4**, 293–302.

119 Bates, J.M., Akerlund, J., Mittge, E., and Guillemin, K. (2007) *Cell Host Microbe*, **2**, 371–382.

120 Goldberg, R.F., Austen, W.G., Jr., Zhang, X., Munene, G., Mostafa, G., Biswas, S., McCormack, M., Eberlin, K.R., Nguyen, J.T., Tatlidede, H.S., Warren, H.S., Narisawa, S., Millan, J.L., and Hodin, R.A. (2008) *Proc. Natl Acad. Sci. USA*, **105**, 3551–3556.

121 Hughes, D.T., and Sperandio, V. (2008) *Nat. Rev. Microbiol.*, **6**, 111–120.

122 Fujiya, M., Musch, M.W., Nakagawa, Y., Hu, S., Alverdy, J., Kohgo, Y., Schneewind, O., Jabri, B., and Chang, E.B. (2007) *Cell Host Microbe*, **1**, 299–308.

123 Peltekova, V.D., Wintle, R.F., Rubin, L.A., Amos, C.I., Huang, Q., Gu, X., Newman, B., Van, O.M., Cescon, D., Greenberg, G., Griffiths, A.M., St George-Hyslop, P.H., and Siminovitch, K.A. (2004) *Nat. Genet.*, **36**, 471–475.

124 Vavricka, S.R., Musch, M.W., Chang, J.E., Nakagawa, Y., Phanvijhitsiri, K., Waypa, T.S., Merlin, D., Schneewind, O., and Chang, E.B. (2004) *Gastroenterology*, **127**, 1401–1409.

125 Merlin, D., Steel, A., Gewirtz, A.T., Si-Tahar, M., Hediger, M.A., and Madara, J.L. (1998) *J. Clin. Invest.*, **102**, 2011–2018.

126 Buyse, M., Tsocas, A., Walker, F., Merlin, D., and Bado, A. (2002) *Am. J. Physiol. Cell Physiol.*, **283**, C1795–C1800.

127 Merlin, D., Si-Tahar, M., Sitaraman, S.V., Eastburn, K., Williams, I., Liu, X., Hediger, M.A., and Madara, J.L. (2001) *Gastroenterology*, **120**, 1666–1679.

128 Fritz, J.H., Le, B.L., Magalhaes, J.G., and Philpott, D.J. (2008) *Trends Immunol.*, **29**, 41–49.

129 Rakoff-Nahoum, S., and Medzhitov, R. (2008) *Mucosal. Immunol.*, **1** (Suppl. 1), S10–S14.

130 Collier-Hyams, L.S., and Neish, A.S. (2005) *Cell Mol. Life Sci.*, **62**, 1339–1348.

131 Rakoff-Nahoum, S., Paglino, J., Eslami-Varzaneh, F., Edberg, S., and Medzhitov, R. (2004) *Cell*, **118**, 229–241.

132 Vijay-Kumar, M., Sanders, C.J., Taylor, R.T., Kumar, A., Aitken, J.D., Sitaraman, S.V., Neish, A.S., Uematsu, S., Akira, S., Williams, I.R., and Gewirtz, A.T. (2007) *J. Clin. Invest.*, **117**, 3909–3921.

133 Vijay-Kumar, M., Wu, H., Aitken, J., Kolachala, V.L., Neish, A.S., Sitaraman, S.V., and Gewirtz, A.T. (2007) *Inflamm. Bowel. Dis.*, **13**, 856–864.

134 Rakoff-Nahoum, S., Hao, L., and Medzhitov, R. (2006) *Immunity*, **25**, 319–329.

135 Medzhitov, R. (2007) *Nature*, **449**, 819–826.

136 Lee, J., Gonzales-Navajas, J.M., and Raz, E. (2008) *Semin. Immunopathol.*, **30**, 3–9.

137 Cario, E., and Podolsky, D.K. (2000) *Infect. Immun.*, **68**, 7010–7017.

138 Fernandes-Alnemri, T., Yu, J.W., Datta, P., Wu, J., and Alnemri, E.S. (2009) *Nature*, **458**, 509–513.

139 Lee, M.S., and Kim, Y.J. (2007) *Annu. Rev. Biochem.*, **76**, 447–480.

140 Girardin, S.E., Boneca, I.G., Carneiro, L.A., Antignac, A., Jehanno, M., Viala, J., Tedin, K., Taha, M.K., Labigne, A., Zahringer, U., Coyle, A.J., DiStefano, P.S., Bertin, J., Sansonetti, P.J., and Philpott, D.J. (2003) *Science*, **300**, 1584–1587.

141 Girardin, S.E., Boneca, I.G., Viala, J., Chamaillard, M., Labigne, A., Thomas, G., Philpott, D.J., and Sansonetti, P.J. (2003) *J. Biol. Chem.*, **278**, 8869–8872.

142 Viala, J., Chaput, C., Boneca, I.G., Cardona, A., Girardin, S.E., Moran, A.P., Athman, R., Memet, S., Huerre, M.R., Coyle, A.J., DiStefano, P.S., Sansonetti, P.J., Labigne, A., Bertin, J., Philpott, D.J., and Ferrero, R.L. (2004) *Nat. Immunol.*, **5**, 1166–1174.

143 Hugot, J.P., Chamaillard, M., Zouali, H., Lesage, S., Cezard, J.P., *et al.* (2001) *Nature*, **411**, 599–603.

144 Ogura, Y., Bonen, D.K., Inohara, N., Nicolae, D.L., Chen, F.F., Ramos, R., Britton, H., Moran, T., Karaliuskas, R., Duerr, R.H., Achkar, J.P., Brant, S.R., Bayless, T.M., Kirschner, B.S., Hanauer, S.B., Nunez, G., and Cho, J.H. (2001) *Nature*, **411**, 603–606.

145 Maeda, S., Hsu, L.C., Liu, H., Bankston, L.A., Iimura, M., Kagnoff, M.F., Eckmann, L., and Karin, M. (2005) *Science*, **307**, 734–738.

146 Brandl, K., Plitas, G., Schnabl, B., DeMatteo, R.P., and Pamer, E.G. (2007) *J. Exp. Med.*, **204**, 1891–1900.

147 Bergsbaken, T., Fink, S.L., and Cookson, B.T. (2009) *Nat. Rev. Microbiol.*, **7**, 99–109.

148 Fritz, J.H., Girardin, S.E., Fitting, C., Werts, C., Mengin-Lecreulx, D., Caroff, M., Cavaillon, J.M., Philpott, D.J., and dib-Conquy, M. (2005) *Eur. J. Immunol.*, **35**, 2459–2470.

149 Zaph, C., Troy, A.E., Taylor, B.C., Berman-Booty, L.D., Guild, K.J., Du, Y., Yost, E.A., Gruber, A.D., May, M.J., Greten, F.R., Eckmann, L., Karin, M., and Artis, D. (2007) *Nature*, **446**, 552–556.

150 Lee, J., Mo, J.H., Katakura, K., Alkalay, I., Rucker, A.N., Liu, Y.T., Lee, H.K., Shen, C., Cojocaru, G., Shenouda, S., Kagnoff, M., Eckmann, L., Ben-Neriah, Y., and Raz, E. (2006) *Nat. Cell Biol.*, **8**, 1327–1336.

151 Kelly, D., Campbell, J.I., King, T.P., Grant, G., Jansson, E.A., Coutts, A.G., Pettersson, S., and Conway, S. (2004) *Nat. Immunol.*, **5**, 104–112.

152 Yuk, M.H., Harvill, E.T., Cotter, P.A., and Miller, J.F. (2000) *Mol. Microbiol.*, **35**, 991–1004.

153 Legarda, D., Klein-Patel, M.E., Yim, S., Yuk, M.H., and Diamond, G. (2005) *Cell Microbiol.*, **7**, 489–497.

154 Tien, M.T., Girardin, S.E., Regnault, B., Le, B.L., Dillies, M.A., Coppee, J.Y., Bourdet-Sicard, R., Sansonetti, P.J., and Pedron, T. (2006) *J. Immunol.*, **176**, 1228–1237.
155 Neish, A.S., Gewirtz, A.T., Zeng, H., Young, A.N., Hobert, M.E., Karmali, V., Rao, A.S., and Madara, J.L. (2000) *Science*, **289**, 1560–1563.
156 Rytkonen, A., and Holden, D.W. (2007) *Cell Host Microbe*, **1**, 13–22.
157 Kumar, A., Wu, H., Collier-Hyams, L.S., Hansen, J.M., Li, T., Yamoah, K., Pan, Z.Q., Jones, D.P., and Neish, A.S. (2007) *EMBO J.*, **26**, 4457–4466.
158 Kumar, A., Wu, H., Collier-Hyams, L.S., Kwon, Y.M., Hanson, J.M., and Neish, A.S. (2009) *J. Immunol.*, **182**, 538–546.
159 Segain, J.P., de la Raingeard, B.D., Bourreille, A., Leray, V., Gervois, N., Rosales, C., Ferrier, L., Bonnet, C., Blottiere, H.M., and Galmiche, J.P. (2000) *Gut*, **47**, 397–403.
160 Yin, L., Laevsky, G., and Giardina, C. (2001) *J. Biol. Chem.*, **276**, 44641–44646.
161 Kles, K.A., and Chang, E.B. (2006) *Gastroenterology*, **130**, S100–S105.
162 Hooper, L.V. (2009) *Nat. Rev. Microbiol.*, **7**, 367–374.
163 Wirtz, S., and Neurath, M.F. (2007) *Adv. Drug Deliv. Rev.*, **59**, 1073–1083.
164 Cho, J.H. (2008) *Nat. Rev. Immunol.*, **8**, 458–466.
165 Koloski, N.A., Bret, L., and Radford-Smith, G. (2008) *World J. Gastroenterol.*, **14**, 165–173.
166 Petri, W.A., Jr., Miller, M., Binder, H.J., Levine, M.M., Dillingham, R., and Guerrant, R.L. (2008) *J. Clin. Invest.*, **118**, 1277–1290.
167 Mann, J.F., Acevedo, R., Campo, J.D., Perez, O., and Ferro, V.A. (2009) *Expert. Rev. Vaccines.*, **8**, 103–112.
168 Belyakov, I.M., Ahlers, J.D., Nabel, G.J., Moss, B., and Berzofsky, J.A. (2008) *Virology*, **381**, 106–115.
169 He, B., Xu, W., Santini, P.A., Polydorides, A.D., Chiu, A., Estrella, J., Shan, M., Chadburn, A., Villanacci, V., Plebani, A., Knowles, D.M., Rescigno, M., and Cerutti, A. (2007) *Immunity*, **26**, 812–826.

11
Dynamic Imaging Technologies to Explore Infectious Processes at the Cellular, Tissue and Organ Level

Jost Enninga, Regis Tournebize, Keira Melican, and Agneta Richter-Dahlfors

11.1
Introduction to Imaging of Host–Pathogen Interactions

The old proverb 'seeing is believing' reflects well the promises and challenges of imaging technologies for biological research: peering through microscopes scientists are now able to observe dynamic biological processes with cellular or even subcellular resolution. On the one hand this yields insight into fundamental biological processes with unprecedented detail and precision. On the other hand imaging is prone to subjective interpretation of the observations, as researchers sometimes 'believe' that they are observing specific pre-assumed phenomena. Therefore, imaging requires particular care in choosing the experimental set-up, implementing rigorous and exhaustive controls for the experiments, and interpreting the obtained results in a well-balanced manner. Taking into account these considerations, imaging is extremely powerful in changing the way the underlying logic of biological processes can be monitored and eventually understood at the cellular and molecular level.

In this chapter, we will focus on the use of novel, mainly fluorescence microscopic imaging technologies to study the interactions between bacterial pathogens and their hosts. We will provide an introduction to how imaging is changing the way we investigate host–pathogen interactions via the availability and development of novel microscope hardware, via fluorescent markers and assays, and via the accessibility of novel bioinformatics tools that allow the automated evaluation of a large number of measured images. The major section of this chapter will then outline prominent entry routes traveled by bacterial pathogens during the infection process, and we will highlight how cutting-edge imaging has been used to address the key issues of these events. We will also discuss current trends and future possibilities for improving the diagnostics of bacterial infection via imaging.

During the last decade significant progress has been made in adapting novel light or fluorescence microscopy techniques for the study of bacterial infection. In particular, time-lapse microscopy using inverted fluorescence microscope set-ups at physiological temperatures inside heated temperature boxes has revealed the

Bacterial Virulence: Basic Principles, Models and Global Approaches. Edited by Philippe Sansonetti
© 2010 WILEY-VCH Verlag GmbH & Co. KGaA, Weinheim
ISBN: 978-3-527-32326-5

dynamics of the cellular cross talk between a large number of pathogens and the host [1]. Furthermore, laser scanning confocal microscopy (LSCM) has been used to acquire images of host–pathogen interactions in three dimensions with very high spatial resolution [2]. In addition, rapid confocal microscope systems with inbuilt spinning 'Nipkov Discs' have become commercially available during recent years and they yield three-dimensional (3D) information at a speed that is approximately 10 times faster than via LSCM. Spinning disc confocal microscopy is particularly powerful in following infection events on the cellular level in 3D over time when used in conjunction with highly sensitive back-illuminated electron multiplying CCD cameras giving 10^2–10^3 images per second [3]. Another important development has been the availability of non-linear multi-photon fluorescent microscopes which give 3D information about underlying tissues up to a depth of 400 µm. This technology will be described in more detail in the section on urinary infection. Together, the available light and fluorescence microscopes have become very powerful instruments for monitoring the dynamics of host infection by pathogens. Currently, a new generation of microscopes is being developed that breaks the resolution limit of light and that will reveal infection processes with molecular resolution in the future (see last section of this chapter).

A major reason for the upsurge in fluorescence microscopy has been the development of fluorescent sensors that label specific molecular and cellular structures [4]. The advantage of fluorescence is its high sensitivity which allows detection of a few fluorescent molecules inside complex sample volumes. Biomolecules, such as nucleic acids or proteins can be labeled in different ways, either via the binding of fluorophores linked to small organic molecules that have high affinity for specific proteins or multi-protein complexes (e.g. rhodamine–phalloidin that binds to F-actin). Another labeling procedure has revolutionized fluorescent imaging of biologic processes: this has been the identification and biotechnological improvement of naturally-occurring, genetically-encoded fluorescent or bioluminescent proteins, such as the green fluorescent protein (GFP) or firefly luciferase [4]. Genetic engineering allows the generation of virtually any protein of interest to be fused to a genetically-encoded fluorescent or bioluminescent marker. Furthermore, stable monomeric fluorescent proteins have recently been developed which span the entire spectrum of visible light allowing the simultaneous monitoring of different proteins [5]. Also, other genetically-encoded tags are currently being developed to avoid interference of the bulky GFP proteins with the protein function. One of these methods is the two-compound tetracysteine–FlAsH approach which exploits the high affinity of a small metallo-organic molecule (FlAsH) that binds with high affinity to engineered tetracysteine stretches and starts to fluoresce upon complex formation [6]. Apart from simply labeling proteins with specific fluorophores, other properties of such fluorophores have been exploited to monitor biological processes: these include fluorophore stability that can be used to monitor protein dynamics via fluorescence recovery after photobleaching (FRAP), or the transfer of fluorescence between neighboring fluorophores via fluorescence resonance energy transfer (FRET). The current uses of

these techniques which have been described and reviewed extensively, are referred to later in this chapter [3].

Imaging infectious processes rapidly leads to large data-sets that need to be analyzed in an unbiased fashion. This can be achieved via digital processing using a number of commercial programs, or programs that are freely available on the internet. Furthermore, an increasing number of research institutions are recruiting dedicated research groups to focus on the development of image-processing algorithms. Even though commercial microscopes often come with powerful image-analysis software suits (for example, in the case for Leica, Zeiss, Nikon or Olympus), more sophisticated data-analysis programs have more options for individual applications. One such software suit is known as 'Metamorph' (Molecular Devices) which can even be used to drive microscopes and contains a large number of macros to process and quantify the obtained imaging data. Other programs, such as 'Imaris' (Bitplane), 'Volocity' (Improvision), or 'Huygens' (Scientific Volume Imaging, SVI) have more specific applications such as the analysis of multi-dimensional data-sets for the first two, or the de-convolution of data-sets for the third. A large number of image processing and analysis tasks can also be undertaken with freely available programs that can be downloaded from the internet without charge. One example is the program 'ImageJ' that can be upgraded with hundreds to thousands of freely available plug-ins and macros from the internet and represents a very powerful tool for image analysis (a link to the program and these plug-ins can be found at http://rsbweb.nih.gov/ij/). A particular strength of ImageJ is the possibility of further development of the program by individual users via its open source code in the Java language. Also, it is very simple to write new ImageJ plug-ins and macros. Another freely available program is Osirix which facilitates the analysis of multidimensional data-sets originating from a wide variety of source images such as positron emission tomography (PET) scans into three-dimensional confocal slices (can be downloaded from www.osirix-viewer.com). Overall, the choice of program is dictated by the specific application required, however as we pointed out most of the tasks can be addressed by open-source software which is freely available on the internet.

11.2 Imaging of Infections

In this section we will focus on how cutting-edge imaging has been used to monitor and analyze various infectious processes caused by bacterial pathogens. We will give examples of the infection of different organs and the recurrent imaging technologies used for their study. This will enable us to draw parallels between the technological requirements for different infection processes. Also, this shows how specific imaging methods can be applied to study a multitude of infectious diseases.

11.2.1 Airway and Lung Infections

Lungs, with an exposed area of 200 m^2, represent the largest body area in contact with the external environment; however, its distal region remains free of bacteria. Indeed, alveoli are characterized by a thin epithelium lining the endothelium, permitting gas exchange between air and blood. This peculiar structure is very sensitive to infection as a widespread inflammation can lead to tissue destruction and thus complete and fatal impairment of the entire organ. The importance of the lung is exemplified by the fact that respiratory diseases of infectious origin are the primary cause of death worldwide [7]. These infections are mainly caused by a large number of bacteria and viruses, among which many are still unidentified.

A battery of innate immune mechanisms has been selected throughout evolution in order to keep the airways sterile. Normally, inhaled particles do not make their way down to the alveoli whether they are inert (e.g. exhaust particles) or microbes (yeast, bacteria, or viruses). They are trapped within the mucus and transported upwards in the airways by mucocilliary transport. Mucus is a glycoproteic meshwork of secreted mucins lining the conducting airway epithelia whose function is to protect the airways and trap extracellular particles. Mucins are secreted by goblet cells in the human upper airways and by Clara cells in mouse lungs and human distal airways. Ciliated cells push the mucus layer upwards in the airways by the coordinated beating of the cilia thus eliminating trapped particles. In addition, mucus contains a large variety of molecules which specifically target microbes, such as secreted immunoglobulins (IgA), lyzozyme, and antimicrobial peptides, whose expression is enhanced during infection and inflammation [8, 9]. When pathogens or other particles make their way to the alveoli, they are phagocytosed by a larger number of resident macrophages leading to the initiation of an inflammatory response in order to eliminate them.

Pneumonias are characterized by an acute inflammatory reaction featuring an important recruitment of polymorphonuclear cells. Even though in half of the cases, a pathogen cannot be identified, viruses are involved in 11.7% of patients. *Streptococcus pneumoniae* and *Mycoplasma pneumoniae* are the two main bacteria responsible for pneumonia in 19.3 and 11.1% of cases respectively [10–12]. With regard to community-acquired pneumonia in humans, *S. pneumoniae* and *Klebsiella pneumoniae* are the two major causative agents of this type of pneumonia. *S. pneumoniae* is an asymptomatic commensal of the upper airways that can invade the lower respiratory tract and cause pneumonia. *K. pneumoniae* is responsible for acute lobar pneumonia characterized by massive polymorphonuclear cell recruitment to the site of infection. Both bacteria, although very different, share several virulence factors such as capsule and metal-binding proteins [13–18]. Both express a thick capsule which provides substantial anti-phagocytic protection and resistance to complement activity. The negatively-charged capsule of *S. pneumoniae* allows the bacteria to cross the mucus layer and encounter the underlying epithelium. However, as the capsule prevents adhesion to the epithelium, pneumococci

such as *Neisseria meningitidis* undergo phase variation and express a thinner capsule to favor their interaction with cells or extracellular matrix proteins (reviewed in [14]). In contrast, *K. pneumoniae* expresses a neutrally-charged surface that prevents its interaction with host cells [19]. Adhesion molecules are also important virulence factors. They include for instance, phosphorylcholine, choline-binding proteins, pavA and enolase in the case of pneumococci and fimbriae in *K. pneumoniae*. Additional specific virulence factors include pneumolysin in pneumococci and LPS and some metabolic enzymes in *K. pneumoniae* [20–23].

With the exception of bioluminescence (BLI), classical *in vivo* imaging techniques have not been widely used to investigate the pathogenesis of lung infections. Microscopy-based techniques are of limited use for lung imaging because of the difficulties with access and the constant movements of the lungs during breathing. Other imaging techniques such as positron emission tomography (PET) or magnetic resonance imaging (MRI) are not ideal for providing images of the lungs, although they are useful in many aspects. The lungs non-specifically retain many of the PET probes that have been synthesized and therefore rejected, leaving only a few available for lung imaging. Due to the low cell density in the lung and the large interface between the cells and the air that create artifacts which interfere with the signals, MRI is not used extensively to investigate lung morphology and metabolism during infection. This explains why whole body *in vivo* imaging techniques like bioluminescence have been widely used for over 10 years to monitor the fate of pathogens in the context of airways infection in model organisms.

Bioluminescence (BLI) is based on the production of light by bacteria engineered to express luciferase. Bacteria either express the sole luciferase, and in this case, the substrate, luciferin must be injected into the animal to allow production and detection of light. Alternatively, bacteria express a whole operon encoding for luciferase together with the enzymes responsible of the synthesis of the substrate, allowing its direct detection. Bioluminescence has proven to be extremely useful in the study of infectious diseases by revealing or highlighting new dissemination routes. In the case of *Bacillus anthracis*, it was thus possible to show that infectious spores establish infection at the site of inoculation and not in the draining lymph nodes to which they were believed to be transported first [24]. In the case of pneumococcal infection, tagging the bacteria with luciferase allowed the number of bacteria in an *in vivo* model of pneumonia to be tracked and quantified [25, 26]. There are few reports of PET imaging of lung inflammation. Most of these studies detected [18F]fluorodeoxyglucose (FDG), a glucose analog transported into cells that cannot be metabolized. FDG is thus used as a marker of metabolic activity. Endotoxin-induced inflammation in animal models is characterized by a neutrophil influx that can easily be detected by FDG-PET imaging (reviewed in [27]). Similarly, this imaging technique allows the visualization of inflammatory foci caused by *Pseudomonas aeruginosa* in lungs [28]. Although very sensitive, PET provides images of only a few mm in resolution, and is not particularly adaptable to high resolution imaging especially in small animal models. Unlike PET, MRI gives images of higher resolution (in practice around 100 μm), even though it is

less sensitive. Nevertheless, it can be used to detect and visualize endotoxin-induced lung inflammation in rats [29]. In addition, the appearance and regression of inflammation and edema caused by infection with *K. pneumoniae* [30] or *P. aeruginosa* in cystic fibrosis can be monitored in mice by MRI (Figure 11.1) [31]. It would be of interest to combine both imaging modalities for the study of infectious disease, i.e. to take advantage of the high resolution images provided by MRI and the specific and sensitive detection of PET probes. Such dual imaging systems are currently being developed and have proven to provide interesting and complementary information in a model of engrafted tumor in mice [32].

Bronchoscopy is a more invasive technique used in the clinic to visualize the trachea and bronchi directly, but the size of the bronchoscope does not permit exploration of the distal airways in humans. However the recent development of fibered confocal fluorescence microscopy (FCFM) greatly improved *in situ* lung imaging [33]. Fiber optic probes are introduced via a classical bronchoscope and passed further down into the distal lungs and used to visualize human terminal bronchioles directly (see www.maunakeatech.com). This allows the detection of auto-fluorescence from tissue or cells labeled with exogenous dyes. With the constant development of molecular imaging, it is now possible to combine detection of specific probes by FCFM. Cortez-Retamozo *et al.* [34] thus recently visualized eosinophil-associated matrix metalloproteinases activity in inflamed mouse lungs using both fluorescence-mediated molecular tomography and FCFM. However, FCFM only allows imaging of the bronchial walls in mice and not the distal airways. Nevertheless, molecular imaging using MRI, fluorescence tomography, PET, or FCFM holds promising prospects for *in vivo* imaging of infectious processes in animal models, giving simultaneously images of both the pathogen and the host response.

11.2.2
Infections of the Gastrointestinal Tract

Infections of the gastrointestinal (GI) tract are one of the major causes of morbidity and mortality worldwide with about 5 million deaths annually most of which are infant deaths. GI infections occur when pathogens are ingested via spoiled food or water or via feces, suggesting that improvement of sanitation would play a major role in preventing such infections. Therefore, infections are particularly severe in regions with socio-economic problems or in those facing crises such as war. In the industrialized world GI infections are also present, however they are generally treated efficiently mostly via simple re-hydration therapy or via the rapid access to antibiotics. The main genera causing bacterial GI infections are pathogenic Enterobacteriaceae such as *Enterotoxigenic*, *Enteropathogenic*, *Enterohemorragic* and *Enteroinvasive E. coli* (ETEC, EPEC, EHEC and EIEC), *Shigella*, *Salmonella*, *Yersinia* and *Proteus*. Furthermore, bacterial GI pathogens belong to the genus of the Vibrionaceae and include *Vibrio cholerae* or *Campylobacter jejuni*.

A special feature of GI infections is their environment: with regard to colonization by microorganisms, the environment in the GI tract varies from being almost

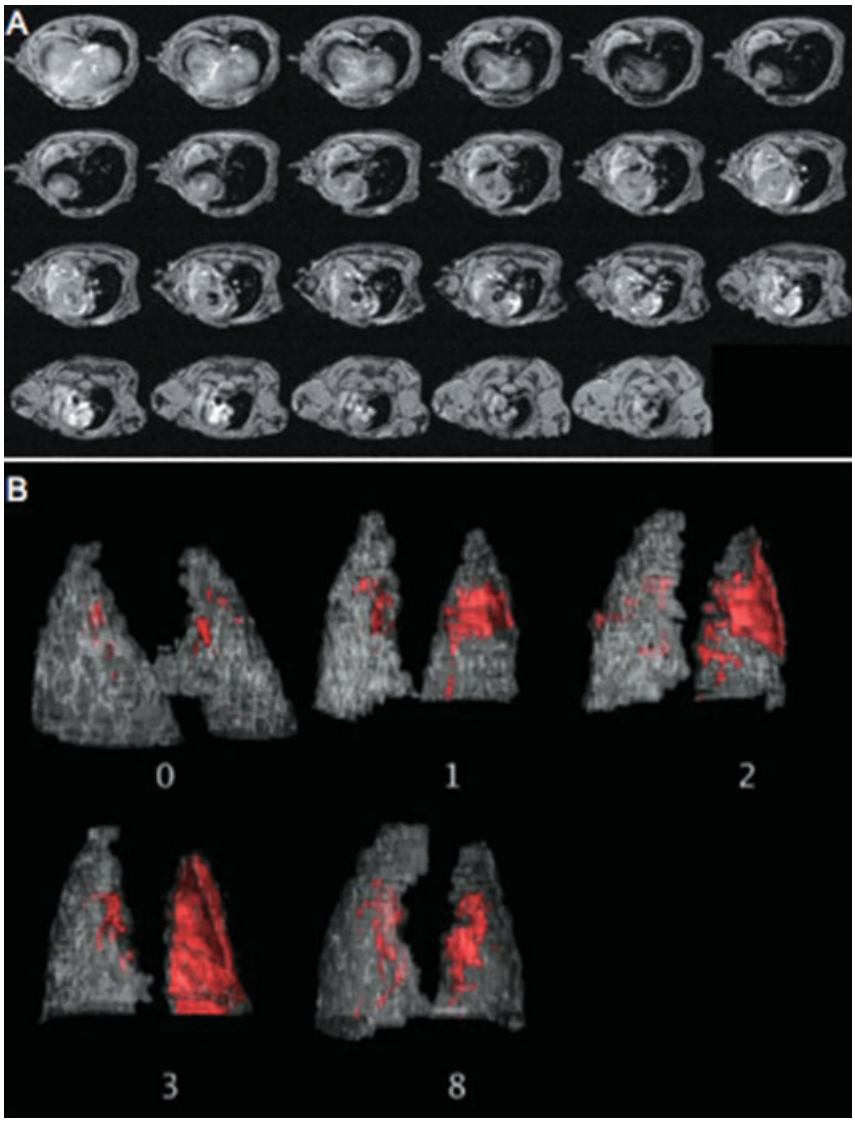


Figure 11.1 MR Imaging of inflammatory lesions in lungs of mice infected with *Klebsiella pneumoniae* and then treated with antibiotics. (A) Serial slices through the thorax of a mouse 3 days post infection and receiving antibiotics. Resolution of the image is 137*137*500µm. These images can be used to reconstruct (B) the inflammatory lesions (red areas) within the whole lungs (gray) in 3D and can be used to follow the development and regression of inflammation in a spatio-temporal manner. Numbers indicate days post-infection.

aseptic in its proximal region (about 10/ml microorganisms within the small intestine), then becoming increasingly rich in Gram-positive flora, coliforms and anaerobic bacteria, and finally in its distal region becoming colonized by the highest numbers of microorganisms found within the human body (more than 10^9/ml microorganisms inside the colon). Among the flora found in the GI tract of healthy individuals there are about 400 different bacterial species which maintain a symbiotic relationship with the host [35]. Therefore, GI infections and their proper treatment should be seen in the context of the naturally-occurring gut flora as imbalances among these symbiotic organisms can enhance GI infections or mimic their symptoms.

Bacterial GI infections lead to a large variety of symptoms depending on the pathogen. Generally, watery or secretory diarrhea is distinguished from bloody diarrhea and dysentery. Secretory diarrhea leading to fluid and electrolyte loss may have a mild course (as is the case for various EPEC strains or *Campylobacter* infections), or it can be severe and life threatening as in the case of cholera (caused by *Vibrio cholerae*). In contrast, bloody diarrhea and dysentery represent more severe diseases leading to the destruction of parts of the GI tract and massive inflammation. These are mainly caused by *Shigella*, *Salmonella* and EHEC. In addition, these infection can spread to other parts of the human body, as is the case during typhoid fever (caused by *Salmonella typhii* or *paratyphii*), or lead to osteomyelitis (caused by *Salmonella typhimurium*), or even trigger life-threatening complications such as sepsis that may occur after perforation of the colon during infection [36, 37]. Generally, bloody diarrhea and dysentery require antibiotic therapy to eliminate the pathogen as rapidly as possible. Often, it is difficult to differentiate certain forms of bloody diarrhea, dysentery and watery diarrhea, for example *Shigella sonnei* infections can produce symptoms similar to those caused by EPEC. Therefore, laboratory diagnostics are necessary to precisely identify the causing bacterial agent in the patient's stool or vomit, or in samples of the spoilt food. Such diagnostics do not generally take advantage of imaging techniques. Only in cases of prolonged chronic symptoms of GI infections is endoscopy carried out to investigate the precise localization of the infected site.

After ingestion, GI pathogens have to withstand the harsh environment of the stomach (low pH) and small intestine (high pH and bile salts) to arrive in and colonize the gut. Therefore, generally high doses of pathogens are required to manifest disease, and only a few examples of GI pathogens that are virulent in low numbers are known. This is the case for *Shigella* which causes infection at doses lower than 100 bacteria [38]. The pathogens causing watery diarrhea are usually non-invasive and remain inside the GI lumen and cause disease via colonization or via the secretion of specific toxins that irritate the host cellular epithelium. To colonize the gut lumen, these pathogens use a large number of specific pili, fimbriae or other bacterial adhesins (reviewed in [39]). For example, *Vibrio cholerae* use the toxin-co-regulated pilus (TCP) to form micro-colonies, and ETEC and EPEC use the colonization factor antigen (Cfa) or the bundle-forming pilus (Bfp1) respectively to attach to the epithelial cell surface. Furthermore, a large number of pathogens secrete toxins via specialized secretion machineries. These

toxins are either secreted into the GI lumen or the pathogens inject so-called effector proteins directly into host cells as will be described in more detail below.

Injection of effector proteins is a common strategy used by GI pathogens that cause bloody diarrhea or dysentery, e.g. *Shigella* and *Salmonella*. These pathogens secrete a cocktail of effector proteins directly into targeted cells via a dedicated type III secretion (T3S) system that in simplified terms, works like a molecular syringe [40]. The common feature of T3S systems is their capacity to translocate bacterial effectors directly from the bacterial cytoplasm into the host cells via three membranes, the bacterial outer and inner cell membrane and the host cellular membrane. Imaging technologies have been instrumental in determining the function of these secretion devices and the roles of the secreted effector proteins. *Shigella* is not able to target epithelial cells from the luminal side of the GI tract, it therefore traverses the M cells within the colon and injects its effectors into the epithelial cells from the basolateral side [38]. *Salmonella* on the other hand, is able to target the epithelium directly from the colon lumen [41]. Injection of the T3S effectors leads to an uptake of both of these pathogens into the cells, and after uptake *Salmonella* evades the host immune system and remains within a vacuolar membrane-bound compartment [41]. In contrast, *Shigella* ruptures the endocytic vacuole and spreads from cell to cell by propelling itself via the formation of actin comet tail at one of its poles [38]. Eventually, this spread leads to epithelial disruption and bloody diarrhea. Furthermore, *Shigella* kills the macrophages that arrive at the site of infection thus inducing apoptosis in a T3S system-dependent manner. During all these processes a massive inflammatory immune response is triggered which leads to the fragmentation of the epithelial barrier and allows more pathogens to cross from the luminal side [38]. *Salmonella* infection does not lead to such a dramatic pro-inflammatory immune response. However, it has been hypothesized that the capacity of *Salmonella* to induce pro inflammatory signaling is correlated with the manifestation of either gastroenteritis or typhoid fever [37].

The complex interplay between GI pathogens and the host highlights the need for technologies with the capacity to track bacterial infection with high spatiotemporal resolution to determine the orchestration of the subsequent events [3]. Imaging has been used extensively to monitor GI infections on the subcellular, cellular and whole-body level. At the whole-body level, the GI tract is not easily accessible for *in vivo* imaging studies of infection with the cellular resolution that is achievable for urinary tract infections as described below. The main reasons for this are the following: first, the constant peristaltic movements of the GI tract prevent focusing even during short time periods (this is also a problem for lung imaging as mentioned above). Second, the GI mucus is highly auto-fluorescent. Third, the distal regions of the gut are increasingly anaerobic and this interferes with the fluorescence of commonly used markers such as GFP which require oxygen to fluoresce properly. However, whole body imaging of GI infections has been used to follow bacterial spread throughout the animal body at lower resolution using BLI [42]. Importantly, these studies carried out on *Salmonella typhimurium* to monitor the bacterial spread during infection were the first studies to apply BLI to *in vivo* microscopy of an infectious disease [43]. Another study using BLI

has shown the importance of the cecum in an infection model of the GI pathogen *Citrobacter* [44].

Imaging technologies have been widely used to investigate the dynamic cross talk between GI pathogens and the host at the cellular level. Such studies have uncovered a number of strategies used by the pathogens, characterized the invasion into host cells during infection, and tracked the host cellular responses during the invasion process. A large number of these studies have employed *Shigella*, *Salmonella*, *Yersinia*, or EPEC as model pathogens, however the approaches developed for these bacteria can also be applied to the study of pathogens other than those which infect the GI tract [39]. In particular, the creative use of fluorescent markers as molecular sensors has played an important role in these studies. This is highlighted by the subsequent examples concerning the secretion of bacterial effector proteins, the localization of invasive GI pathogens within host cells and the induction of host responses after contact with host cells.

Direct translocation of bacterial effectors into host cells via the T3S of *Yersinia* was first shown via a biochemical reporter system measuring the activity of the effector YopE linked to adenylate cyclase [45]. *Yersinia* does not induce its uptake via the secretion of T3S effectors, but uses this device to undermine the host immune system. To establish a direct correlation between the secretion of effectors and changes induced within the host cells would require these events to be monitored in space and real time. It was found that fluorescent labeling of T3S effectors to generate chimeras with GFP blocked their secretion through the T3S needles and hampered the secretion of other effectors (unpublished data). To circumvent these difficulties, three independent strategies for following T3S activity upon contact with host cells in real time have so far been developed by different research groups. The first approach used a FRET reporter consisting of a cephalosporin derivate with a FRET donor linked to one extremity and a FRET acceptor linked to the other [46]. Cells can be loaded with this FRET probe which loses its activity after cleavage by beta-lactamase. To monitor the passage of T3S effectors into host cells via this method, the host cells are first loaded with the FRET probe and type III effectors are fused to beta-lactamase. The injected effector–beta-lactamase chimeras cleave the FRET probe after their translocation into the host cytosol, and the change in fluorescence emission is then monitored [46]. This revealed that different effectors secreted by EPEC show characteristic secretion kinetics of the effectors EspF, EspG, EspH, EspZ, Map, and Tir [47]. A second approach used the property of T3S effectors to bind to bacterial chaperones before their translocation [48]. For this technique, the *Salmonella* effector chaperone invB was fused to GFP and host cells were stably transfected with this protein chimera. Afterwards, these cells were challenged with *Salmonella*, and the translocated effector SipA molecules could be localized within the host cell by their binding to invB-GFP. Quantification of the fluorescent signals revealed that SipA secretion started about 90 s after host cellular contact and lasted for up to 10 min after secretion initiation. Another approach used the direct labeling of T3S effectors via the tetracysteine-FlAsH labeling procedure (see Figure 11.2 for a detailed description of this approach) [6]. These complexes are smaller than the bulky GFPs and do not

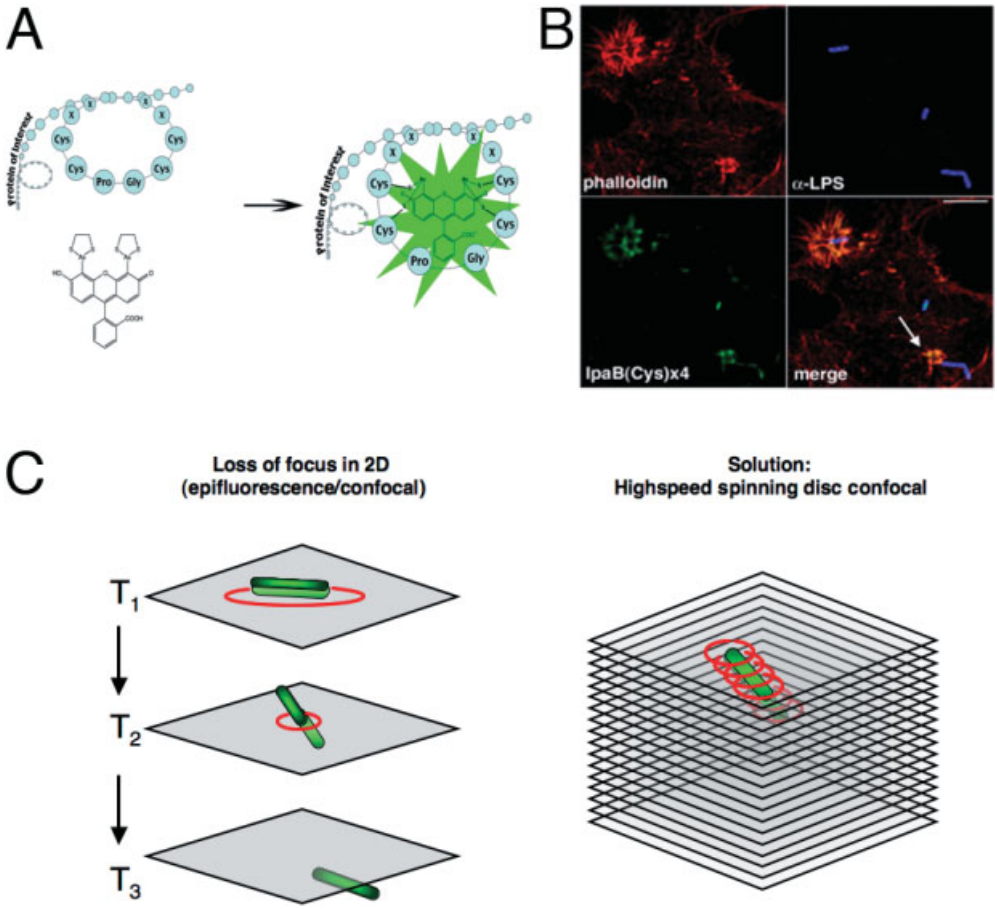


Figure 11.2 (A) Schematics of the 4Cys–FlAsH labeling approach for fluorescently labeling type III effectors inside bacteria without functional loss. The metallo-organic FlAsH compound forms a high-affinity complex with 4Cys sequences that can be added to the effectors. (B) 4Cys-tagged and FlAsH-labeled effectors are secreted from invading *Shigella* (green). The bacteria have been labeled with anti-LPS (blue), and the actin foci are highlighted by phalloidin–rhodamine (red). (C) Set-up for tracking the secretion of fluorescent effectors (via the 4Cys–FlAsH labeling approach) from single bacteria. During the time-course of the experiment, a deep volume needs to be observed because the events are taking place in three dimensions. The fluorescence intensities can then be integrated from single bacteria and correlated with the remaining pool of fluorescent effectors within the bacteria.

impede their secretion via the T3S system. This method visualized the secretion of the *Shigella* T3S effectors IpaB and IpaC from individual bacteria into host cells [49]. This indicated that the effectors are secreted rapidly and concomitantly after contact with the host cells. Furthermore, tetracysteine-FlAsH has been improved

to monitor T3S and used to study the secretion of Salmonella effectors demonstrating that the secretion of two *Salmonella* effectors SopE2 and SptP follows different kinetics [50].

GI pathogens causing dysentery or bloody diarrhea are often internalized into normally non-phagocytotic cells where they hijack the host cellular membrane trafficking machinery and cytoskeleton during these processes. *Salmonella* stays within a *Salmonella* containing vacuole (SCV), and high-speed confocal microscopy has been used to determine the interactions between the SCV and host membrane compartments such as late endosomes and lysosomes [51]. This study revealed that the SCV was in contact with markers of the late endosomal compartment rapidly after its uptake thus challenging the paradigm that this pathogen blocks these events [51]. A similar study has used a number of pathogens such as *Listeria*, *Salmonella* or *Shigella* to assess the role of clathrin during cell entry [52]. These studies indicated that during cell entry there was an elevated concentration of clathrin in close proximity to bacteria such as *Listeria* but that clathrin was not involved in the internalization of other bacteria such as *Salmonella* and *Shigella*. Currently, multiple studies are under way to evaluate and re-evaluate the spatio-temporal hierarchies of host cellular events using rapid confocal microscopy. The results from these studies will either reiterate or challenge the previous findings based on other approaches such as genetics or biochemistry. Eventually, combined research approaches which take into account information from imaging studies using different approaches will lead to a precise understanding of the sequential events during pathogen–host cellular cross talk.

Fluorescent sensors based on FRET have been used to investigate the host responses during GI pathogen invasion. For example, one study monitored the assembly of the components of the major histocompatibility complex (MHC) II during *Salmonella* invasion [53]. This study showed that *Salmonella* is capable of inhibiting the proper assembly of the MHCII complex as a potential mechanism for evading the immune system. The same group has also carried out a high-content/high-throughput study using automated microscopy and RNAi screening to identify the host kinases involved in intracellular *Salmonella* growth [54]. This study described how inhibiting the host kinase AKT1 can counteract its targeting by the *Salmonella* effector SopB to promote bacterial propagation. Automated microscopy, combined with genome or subgenome-wide screens and smart assay development will be very valuable for comprehensive studies on the orchestration of host–pathogen interactions at the cellular and subcellular level.

11.2.3
Urinary Tract Infections

Community-acquired bacterial infection of the human urinary tract occurs when intestinal flora invade and colonize this otherwise sterile environment [55]. Due to anatomical differences adult woman are more susceptible to these infections than adult men with 20–30% of women experiencing recurrent urinary tract infections (UTI) [56]. *Escherichia coli* is considered to be the primary urinary pathogen

and is thought to be responsible for up to 80% of community-acquired infections [57]. Other bacteria associated with UTI include the Gram-negative species *Klebsiella*, *Enterobacter* and *Pseudomona* with *Proteus mirabilis* accounting for more than 40% of infections in infant boys and the Gram-positive organisms, *Staphylococcus epidermidis* and *aureus* and *Enterococcus faecalis* [57].

The urinary tract has numerous inbuilt defense mechanisms such as the flow of urine, low pH, and antibacterial peptides to help maintain sterility [56]. Uropathogenic *Escherichia coli* (UPEC) express a number of virulence factors thought to be important in overcoming a number of these mechanisms and promoting bacterial adaptation to the host. Certain serotypes of *E. coli* such as the somatic O1, O2, O4, O6, O7 and O75 and the capsular K1, K2, K3, K5, K12 and K13, are associated with urinary tract infection and differ from those associated with gastro-intestinal infections [56]. Attachment organelles, such as the Type 1 and P fimbriae allow the pathogen to adhere to the mucosal membranes and withstand the flow of urine. Secreted toxins such as α-hemolysin and cytotoxic necrotizing factor 1 (CNF-1) are also associated with UPEC strains. Despite substantial research into UPEC virulence factors, no one factor has been found to be unique to or characteristic of UPEC, and there is speculation that up to half of the known UPEC isolates contain none or only one of the currently known virulence factors [58]. This variance in virulence factor expression also implicates the role of the host response in the establishment of infection. Disparity in secretor status and expression levels of receptors such as the IL-8 receptor CXCR1 have been shown to influence host susceptibility to UTI [59, 60].

Initial colonization of the urinary tract occurs in the urethra from where the pathogens ascend into the bladder where they may cause asymptomatic bacteriuria or cystitis. The stratified transitional epithelium of the bladder is almost entirely covered with urothelial apical plaques consisting of uroplakin glycoproteins [61]. FimH, responsible for the tip adhesion of the type 1 fimbriae is known to recognize and bind to uroplakins Ia and Ib, allowing UPEC to gain a foothold in this hostile environment [62]. FimH-mediated binding initiates bacterial invasion into the bladder cells which is thought to be the source of recurrent infections [63–65]. Attachment of UPEC to the epithelium also induces an inflammatory response via pattern recognition receptors such as the toll-like receptor pathway (TLR) [66]. UPEC lipopolysaccharide (LPS) is recognized by TLR4-expressing cells and activates the pro-inflammatory NFκB pathway and secretion of chemokines/cytokines. Accumulation of inflammatory chemokines in the urine and bladder mucosa induces the recruitment of neutrophils which aid in the clearance of the infection. Cystitis also induces adaptive immune responses characterized by humoral and cell-mediated responses [67].

Pyelonephritis occurs when infectious pathogens ascend from the bladder into the kidney via the urethra. During ascent of the nephron, UPEC encounter numerous differing environs with the changing composition of primary urine and the expression of antimicrobial peptides by various segments of the nephron. Adhesion of UPEC to renal epithelial cells is thought to differ somewhat from bladder adhesion due to the lack of uroplakin. The tip adhesion of the P fimbriae

is mediated by PapG which attaches to the Galα(α1→4)Galβ-containing glycolipids present on renal epithelium [68]. Kidney epithelium consists of a single layer epithelium, highlighting a crucial difference between kidney and bladder. Upon infection, UPEC trigger a rapid and extensive inflammatory response. After UPEC binding, the kidney triggers a pro-coagulant and pro-inflammatory response which results in rapid vascular isolation of the infection followed by infiltration of immune cells [69]. Infection of the kidney has been associated with more severe inflammation than that which occurs during bladder infection [57]. Abscess formation and edema within the renal parenchyma are often seen with pyelonephritis and lead to the development of irreversible scar formation and can contribute to renal insufficiency [57].

Patients who present with a UTI display numerous symptoms. Lower urinary tract infections often present as pain and urgency of urination. The urine may appear cloudy due to the presence of bacterial, pus and sloughed bladder epithelial cells [56]. Urine examination and culture is essential for diagnosis and the infection is often treated with antibiotics. Upper UTI is more difficult to diagnose because while the symptoms may be similar to those of lower UTI they are often accompanied by a sudden increase in temperature and uni- or bilateral flank pain [70]. Diagnostic tools include standard laboratory examination of blood and urine for the presence of pyuria, bacteriuria, and immune cell casts. In complicated or recurrent cases, diagnostic imaging may be used as a tool to evaluate the extent of damage and any underlying abnormalities that may prevent clearance of the infection. In acute bacterial pyelonephritis, computed tomography (CT) is the preferred imaging method [71].

The diagnostic tools used for patients differ greatly from the state-of-the-art technologies that are available to researchers studying the underlying molecular mechanisms of UTI. Recent advances in imaging technologies combined with a multitude of animal models have allowed researchers to investigate the complex interplay between bacteria and host resulting in the aforementioned pathophysiology. Functional imaging using techniques such as CT and MRI provide information regarding the morphological changes which occur at the organ level following bacterial infection [72]. Imaging techniques based on bioluminescence are also used to study infections at the level of the intact animal. Genetically-engineered uropathogenic bacteria expressing luciferase have been used to monitor the progression and clearance of infection within live UTI models [73]. However, higher resolution is needed to investigate the very early stages of infection when intervention could be crucial to prevent renal damage. Higher resolution imaging can be achieved by confocal microscopy. Confocal microscopy of explanted bladder and kidney has revealed many of the complex interactions that occur between bacteria and the host [74, 75]. Electron microscopy has also been utilized as a means of visualizing at very high resolution, the events that occur in infected tissue such as binding to and invasion of the bladder epithelium [65].

The development of UTI is a very dynamic process and the most relevant environment for visualization of this is within the live animal as it encompasses the full complexity of the infectious environment. Rodent models are the most commonly used *in vivo* UTI models. To enable real-time analysis of tissue events at

cellular resolution in a live animal model, multi-photon microscopy can be used. Multi-photon microscopy differs from confocal imaging in its employment of non-linear techniques which overcome a number of the restraints inherent to confocal microscopy [76]. The multi-photon technique is based around excitation of a fluorophore which occurs due the simultaneous arrival of two photons from a longer wavelength excitation source. The combination of these two photons promotes the fluorophore to its excitation state. This process is mediated by the use of a pulsed laser to alter the concentration of photons in space and time. The technique has a number of advantages for use in living tissue [77]. Longer wavelength excitation allows for greater penetration into light scattering tissues such as the kidney, shorter wavelengths such as those used in confocal excitation can cause a large degree of light scattering and cannot therefore penetrate deep into the tissue. Non-linear microscopy also reduces the amount of photodamage to the tissue, an essential factor for long-term imaging of live tissue [76]. Due to the focused nature of two-photon excitation, only a very small area is excited, meaning that tissue above and below the focal plane are not subjected to photodamage. This very small excitation volume also means that a pinhole is not required to eliminate out-of-focus light emitting from the sample. In multi-photon microscopy detectors are usually set up to capture all the emitted light, allowing for a greater signal to laser power ratio. With less photodamage, multi-photon imaging enables long-term repeat imaging of one area without the damage often seen with confocal microscopy. This means real-time repeat imaging of a single infection site over many hours is now achievable, replacing the need to sacrifice numerous animals at each time point to investigate infection progression [78]. The combination of all these factors means that multi-photon microscopy is very well suited to long-term high resolution imaging of living tissue, a perfect scenario for the study of progression of UTI infections [76–79].

While this technology opens up extensive possibilities for intravital imaging, there are experimental parameters which must be taken into account. Accessibility to the organ can be problematic for deep-set organs and particularly those situated close to the lungs and diaphragm making it difficult to stabilize the organs due to breathing [77]. Although the depth limitations are not as great as those associated with confocal microscopy, there is still room for improvement [80]. The development of optical technology such as the MicroProbe stick objective (Olympus) goes some way to improving accessibility in the animal and has high spatial, temporal, and multi-wavelength resolutions [81]. Other limitations include the use of water immersion optics, limiting resolution, and the speed of acquisition. Due to the scanning nature of multi-photon microscopy the capture speeds are still relatively slow which can be problematic due to movement during 3D stack capture [80]. The use of a live animal model also brings with it the complications involved in maintaining true physiological conditions. Anesthesia levels, body temperature and fluid levels are just a few of the physiological factors which must be monitored and maintained and this requires a wide range of expertise [82]. Much research is underway to address a number of these issues but until definitive results are obtained these issues must still be taken into account during experimental planning.

Multi-photon-based intravital microscopy has been utilized to study numerous tissues and organs with a great deal of pioneering work having been performed in the brain [83]. This work has led to new understanding of a number of neuronal disease processes such as Alzheimer's disease [84]. Advances in the understanding of kidney function have also been achieved with this technique, including new insights into glomerular filtration [80, 85] and albumin uptake [86] in the living tissue. Immunologists have utilized the technique to look at the dynamics of dendritic and T cell interactions within lymph nodes [87], among other aspects of the immune response. The field of infectious diseases is starting to adopt these techniques to study the dynamics of infection within the most physiologically relevant context. In a study that bridges together immunology and infection biology, multi-photon microscopy was used to investigate how infection induced an increase in dendritic cell extensions into the terminal ileum [88].

To study UTI and particularly the earliest stages of pyelonephritis, multi-photon microscopy has been combined with micropuncture delivery of the pathogen directly into the kidney tubule lumen, allowing for a living model with exceptional spatial-temporal resolution (see Figure 11.3a,b for details). A single copy of GFP^+

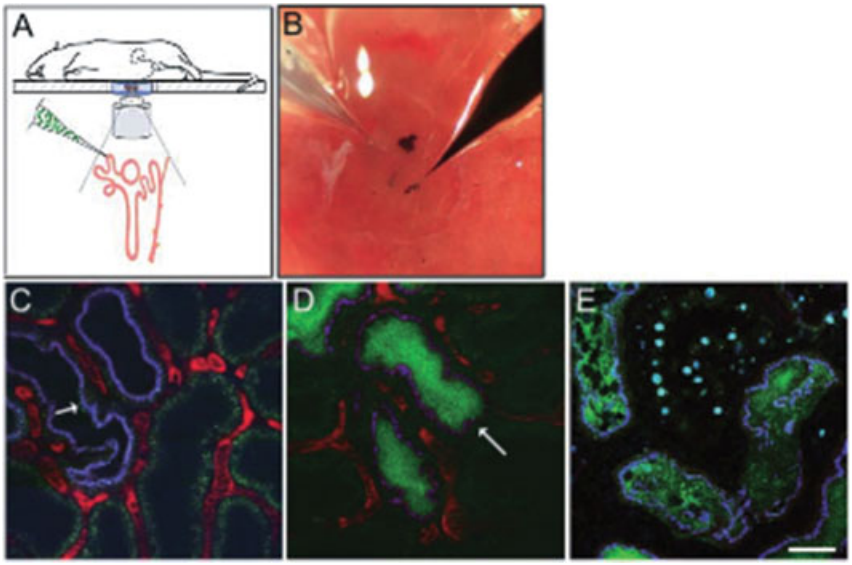


Figure 11.3 Multi-photon microscopy of UTI. (A) Cartoon of the experimental set-up. (B) Micropuncture delivery of bacteria (light blue) and mineral oil (dark blue), which is used to locate the injection site into superficial tubules. (C) At 1 h post injection, a few bacteria (arrow) are seen binding to the proximal tubule. Blood plasma is labeled with a large molecular weight dextran (red), the proximal tubule injected with the dextran has endocytosed the smaller co-injected dextran (blue). (D) At 5 h post injection bacteria (bright green) had filled the treated tubule. The blood flow (red) is showing signs of dysfunction (arrow). (E) At 8 h post injection, bacteria (green) have filled the tubules and all blood flow to the area has stopped (scale bar = 30 μm).

engineered onto the chromosome of the pathogen under the control of a constitutively active promoter has been shown to be sufficient for visualization of single bacteria within the tissue, highlighting the resolution levels of this technique [78]. While the pathogens may be visualized by the use of recombinant proteins, visualization of the tissue is also necessary. Due to the fluorescent nature of the imaging, fluorescent dyes and markers must be used to label the tissue structure without overtly affecting its structure and function. The use of different sizes of labeled dextrans has been utilized extensively in studies on the kidney. Large molecular weight dextrans (500 kDa) are too large to be filtered through the glomerulus and are thus retained within the vasculature, allowing for visualization of the renal capillaries as well as the quantitative analysis of the flow rate of the red blood cells [80]. Smaller dextrans (10 kDa) can be used to label the organ structure under study, as small dextrans are specifically taken up by the proximal tubule epithelial cells in the nephron (Figure 11.3c, d) [78]. The use of regular antibody staining is limited by the physiological effects it may have on the cellular responses. Dyes such as Hoechst 33342 may be used in shorter term studies but are unsuitable for use over the long term because of their DNA binding properties. Delivery of dyes is usually achieved by intravenous injection so concentrations and toxicity need to be calculated for the entire body mass.

This work has given new insight into the speed of the host's response to infection, showing infiltration of immune cells and other physiological responses within the first 3–5 h (Figure 11.3c–e). The intravital nature of this method also allows for the study of parameters which are not measurable in other models e.g. blood and filtrate flow. The rapid effect of epithelial infection on the local vasculature was one such finding. It was found that immediately following bacterial–host interaction, the epithelium increased its oxygen consumption while up-regulating a number of cytokines. These cytokines may be the trigger for endothelial activation leading to clotting and vascular shutdown. This response was shown to be essential for the prevention of bacterial dissemination into the bloodstream and fatal sepsis [69]. The effect of filtrate flow on bacterial binding and colonization has also been investigated and the role that certain binding factors play in colonization has been demonstrated (unpublished data). Collectively this data has shown how the use of dynamic intravital imaging has revealed a number of new factors, both bacteriological and physiological, in the progression of pyelonephritis, a well-established and well-studied infectious process.

11.2.4 Meningitis

Many pathogens of the lungs and urinary or intestinal tracts can disseminate into the blood, cross the blood–brain barrier and then cause meningitis. This is the case for *S. pneumoniae*, *N. meningitidis*, *L. monocytogenes* and some pathovars of *E. coli*. However, how these bacteria cross the blood–brain barrier remains poorly understood. Adhesion of *N. meningitidis* to endothelial cells was investigated by determination of the shear stress level in the brain microcirculation [89]. Using

video-microscopy in a flow chamber, Mairey *et al.* [89] observed that the bacteria adhere to endothelial cells when shear forces are lower than those found during normal blood flow. This study also addressed the question of meningococcal adhesion to the brain endothelium. By imaging brain capillaries through a cranial window using confocal microscopy, it was demonstrated that some capillaries transiently present a decreased blood flow and thus low shear stress levels which enable the meningococci to bind to the endothelium. Once bound, the bacteria irreversibly adhered to cells, proliferated and then traversed the endothelial cells.

Listeria use different strategies to cross the blood–brain barrier (reviewed in [90]) and cause meningitis. Free bacteria found in the blood can directly invade and cross the endothelial cells. Alternatively, they can migrate centripetally from invaded peripheral neurons. The third 'Trojan horse' strategy is to hide within specific cell populations. The so-called inflammatory monocytes, or Ly6C+ monocytes are a subset of monocytes which are involved in the eradication of several types of pathogens such as *Toxoplasma gondii*, *Aspergillus nidulans*, and *L. monocytogenes* [91, 92]. These cells have been shown to cross the blood–brain barrier in mice and transport intracellular *Listeria* from the blood to the brain where they escape the inflammatory monocytes and go on to cause meningitis [93, 94].

Although *E. coli* is most commonly associated with gastrointestinal and urinary tract infections, it is also a common causative agent of meningitis in the newborn. Where and how the bacterium crosses the epithelial lining in the urinary tract to gain access to the bloodstream is currently unknown. *E. coli* meningitis is most commonly associated with the capsular K1 serotype which is also associated with UPEC strains.

11.3 Clinical Implications

The data produced by these dynamic imaging technologies provides a previously unprecedented view of the complexities of the events which occur during the progression of infection. By combining this imaging with both traditional and emerging molecular techniques which can confirm and quantify findings from visualization experiments, a more complete understanding of the infection process at the tissue and molecular level can be obtained. Real-time visualization techniques can help in the identification of novel targets for drugs as well as the effect of a drug, which can be evaluated in high resolution within the appropriate disease state [76]. Dynamic imaging technologies can also help to provide a greater understanding of the molecular results obtained at the laboratory bench by relating them to the context of the tissue. The use of intact live models has the advantage of observing the infectious process within the entire system. While an infection may originate within a single organ, subsequent effects on other organs as well as systemic infections can also be followed. In the above-mentioned study on UTI [69], it was found that treatment for clotting which was highly localized in the infection site in the kidney, in fact triggered the systemic spread of the pathogen

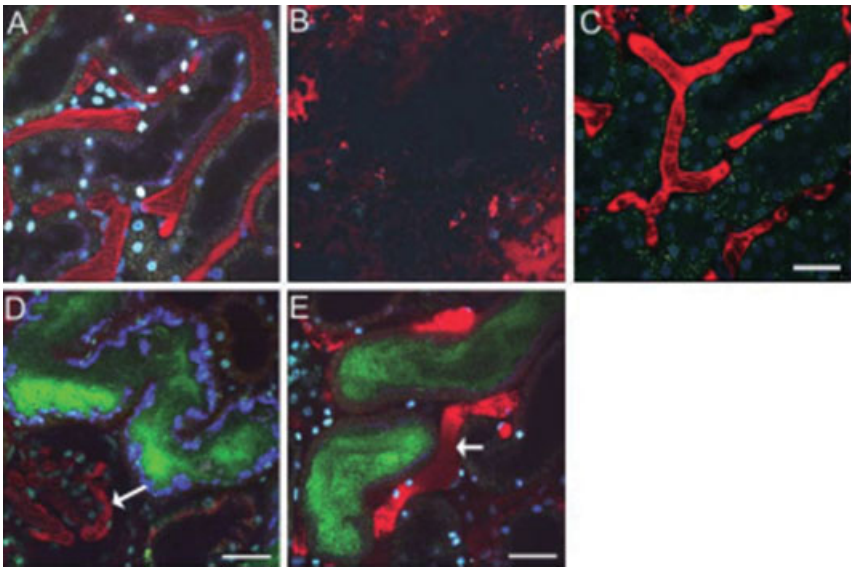


Figure 11.4 (A) Live intravital imaging of injected kidney tubule (blue outline) at 1 h showing little/no sign of bacteria and the free flow of blood (red). (B) Infection site at 22 h post injection. No blood flow, bacteria or tissue morphology remains. (C) Neighboring nephron at 22 h shows normal morphology. (D) Infection at 8 h showing lack of peritubular capillary blood flow but maintenance of glomerular flow (arrow). (E) An infection at 8 h in an animal treated with the anticoagulant heparin sodium shows an extensive volume of plasma streaming into the peritubular capillaries (arrow). All animals treated with heparin sodium suffered fatal sepsis within 9 h.

causing death and leading to high bacterial counts in the heart, liver and spleen (see Figure 11.4 for details). These results demonstrate how findings from imaging may lead to a re-evaluation of clinical procedures. This data indicates that the risks of coagulation in sepsis may need to be weighed against the possibility of anticoagulation therapy which may actually increase bacterial translocation [69]. These findings also demonstrate another great advantage of intravital imaging: the ability to monitor physiological functions within the tissue. As multi-photon intravital microscopy is so well established in the study of renal physiology, it is possible to utilize that expertise to investigate kidney infection. The use of dyes to follow blood flow, filtrate flow in addition to glomerular filtration and cellular endocytosis during infection gives new insight into how and when the pathogenic process may affect these factors. Flow of both blood and primary filtrate through the nephron may be crucial for the delivery of antibiotics to clear the infection. The use of dynamic imaging in the study of bacterial infections in UTI, as well as in other infections can provide a wealth of novel information that will advance our understanding of the infectious process at both the molecular and tissue levels. It holds great possibilities for further advancement in the search for novel regimes of treatment and prevention of many common infections.

11.4 Future Technological Developments

Recently, physicists have attempted to increase the resolution limit of visual light using the equation r = λ/2*N.A., where r is the resolution, λ the wavelength, and N.A. the numerical aperture of the objective. This equation defines the limits of spatial resolution by optical microscopes to about 250 nm. Resolution below this limit is called super-resolution, and the methodologies developed to achieve this use either specific fluorophores or novel optical set-ups for the imaging process [95]. So far, these methodologies have not been applied to the study of bacterial pathogen infections, but have potential for use in such studies in the future. A recently developed approach exploits the stochastic activation of fluorophores via laser pulses. This methodology is known as photoactivated localization microscopy (PALM) or stochastic optical reconstruction microscopy (STORM) [96, 97]. During a PALM or STORM experiment fluorophores are excited repeatedly up to 10 000 times leading to stochastic activation. Images are then extracted from these data-sets at resolutions between 10 and 100 nm. For example, STORM and PALM has been used to obtain high-resolution images of the cytoskeleton and associated proteins. These methods generally use the total internal reflection fluorescence (TIRF) mode of a fluorescence microscope which limits the number of possible applications for the investigation of host–pathogen interactions. A second approach uses two laser beams that are overlaid to illuminate the biological samples. This approach, known as stimulated emission depletion (STED) microscopy requires particular optics because the first laser beam illuminates the second which has a donut-shaped focus that depletes the fluorophores in the excited state thus improving the resolution of the objects [98]. One problem of STED is the increased intensity required to illuminate the biological specimen, and once this is optimized STED will be potentially useful for monitoring host–pathogen interactions due to its unprecedented resolution. In addition to these two examples which appear to be the most suitable for investigation of host–pathogen interactions, other techniques have been and are currently being implemented as detailed in a recent review [95].

Another aspect relevant to the optimization of methods for monitoring host–pathogen interactions is the number of parameters that can be measured simultaneously in a single experiment. In addition, these measurements need to be robust and specific. Hence, the assays used to study of host–pathogen interactions will need to be improved. This will include the direct measurement of the biological parameters of interest. For example, activation of signaling cascades has typically been measured via the translocation of NFκB into the nucleus [99] and this does not yield exact data with regard to which signaling pathway has been activated to induce the translocation. Also, it does not give precise information about which set of genes is regulated by the translocation. Therefore, precise measurements that give information concerning the activation of specific signaling pathways, or that can measure the regulation of the genes that have been identified need to be developed. For example, a number of FRET-based assays are able to measure the activation of a specific signaling molecule as has been reported for the tyrosine

kinase src [100]. Other FRET-based assays have been used to track the activity of small GTPases such as Rac1, and this has been related to the cellular challenge by bacterial pathogens [101]. It will be important to combine many such assays so that information about a maximum number of parameters can be obtained from a single experiment, this will then provide new insight into how different events are connected in living cells.

The third aspect that should be addressed in future research is to relate whole body imaging to cellular and subcellular resolution. The importance of this has been outlined in the section on urinary tract infection. As mentioned in the paragraph above, it will be important to develop approaches that give precise information about specific cellular events. So far, such assays have been mostly developed on the single cell level, but some studies have already obtained results which yield 'functional' read-outs using multi-photon microscopy. One such assay follows the cleavage of a caspase-sensitive probe to measure the rapid onset of apoptosis in real time via biophoton microscopy [102]. Such assays will improve our understanding of cellular processes in the context of living organs and will prove to be very powerful in the study of pathogen infection.

Monitoring urinary tract infections will lead to further breakthroughs in the next few years due to a number of ground-breaking studies in the field of renal physiology. These include the use of fluorescently-labeled albumin to study renal albumin filtration rates *in vivo* [86]. This work revealed filtration rates ~50 times greater than previously measured which led to further debate within the field. The development of techniques using different sizes of fluorescent dextrans and charged molecules has also highlighted the role of intravital imaging in quantifying and qualifying physiological parameters within the living model [103, 104]. Molecular techniques that are already well established in many laboratories, such as the use of siRNA are being developed for use *in vivo* in conjunction with intravital imaging [104]. These studies are bridging the gap between descriptive and quantitative microscopy and will soon provide information from within a living model as opposed to cell culture.

References

1 Enninga, J., Sansonetti, P., and Tournebize, R. (2007) Roundtrip explorations of bacterial infection: from single cells to the entire host and back. *Trends Microbiol.*, **15**, 483–490.

2 Richter-Dahlfors, A., Buchan, A.M., and Finlay, B.B. (1997) Murine salmonellosis studied by confocal microscopy: *Salmonella typhimurium* resides intracellularly inside macrophages and exerts a cytotoxic effect on phagocytes *in vivo*. *J. Exp. Med.*, **186**, 569–580.

3 Frischknecht, F., Renaud, O., and Shorte, S.L. (2006) Imaging today's infectious animalcules. *Curr. Opin. Microbiol.*, **9**, 297–306.

4 Giepmans, B.N., Adams, S.R., Ellisman, M.H., and Tsien, R.Y. (2006) The fluorescent toolbox for assessing protein location and function. *Science*, **312**, 217–224.

5 Shaner, N.C., Campbell, R.E., Steinbach, P.A., Giepmans, B.N., Palmer, A.E., and Tsien, R.Y. (2004) Improved monomeric red, orange and

yellow fluorescent proteins derived from Discosoma sp. red fluorescent protein. *Nat. Biotechnol.*, **22**, 1567–1572.
6 Gaietta, G., Deerinck, T.J., Adams, S.R., Bouwer, J., Tour, O., Laird, D.W., *et al.* (2002) Multicolor and electron microscopic imaging of connexin trafficking. *Science*, **296**, 503–507.
7 Mizgerd, J.P. (2006) Lung infection–a public health priority. *PLoS Med.*, **3**, e76.
8 Schutte, B., and Mccray, P. (2002) [beta]-defensins in lung host defense. *Annu. Rev. Physiol.*, **64**, 709–748.
9 Froy, O. (2005) Regulation of mammalian defensin expression by Toll-like receptor-dependent and independent signalling pathways. *Cell. Microbiol.*, **7**, 1387–1397.
10 Woodhead, M. (2002) Community-acquired pneumonia in Europe: causative pathogens and resistance patterns. *Eur. Respir. J. Suppl.*, **36**, 20s–27s.
11 Garau, J., and Calbo, E. (2008) Community-acquired pneumonia. *Lancet*, **9**, 455–458.
12 Franquet, T. (2001) Imaging of pneumonia: trends and algorithms. *Eur. Respir. J.*, **18**, 196–208.
13 Campos, M.A., Vargas, M.A., Regueiro, V., Llompart, C.M., Albertí, S., and Bengoechea, J.A. (2004) Capsule polysaccharide mediates bacterial resistance to antimicrobial peptides. *Infect. Immun.*, **72**, 7107–7114.
14 Kadioglu, A., Weiser, J.N., Paton, J., and Andrew, P. (2008) The role of *Streptococcus pneumoniae* virulence factors in host respiratory colonization and disease. *Nat. Rev. Microbiol.*, **6**, 288–301.
15 Lawlor, M.S., Hsu, J., Rick, P.D., and Miller, V.L. (2005) Identification of Klebsiella pneumoniae virulence determinants using an intranasal infection model. *Mol. Microbiol.*, **58**, 1054–1073.
16 Lawlor, M.S., O'Connor, C., and Miller, V.L. (2007) Yersiniabactin is a virulence factor for Klebsiella pneumoniae during pulmonary infection. *Infect. Immun.*, **75**, 1463–1472.
17 Nassif, X., and Sansonetti, P.J. (1986) Correlation of the virulence of Klebsiella pneumoniae K1 and K2 with the presence of a plasmid encoding aerobactin. *Infect. Immun.*, **54**, 603–608.
18 Nassif, X., Fournier, J.M., Arondel, J., and Sansonetti, P.J. (1989) Mucoid phenotype of Klebsiella pneumoniae is a plasmid-encoded virulence factor. *Infect. Immun.*, **57**, 546–552.
19 Clements, A., Gaboriaud, F., Duval, J.F., Farn, J., Jenney, A., Lithgow, T., *et al.* (2008) The major surface-associated saccharides of *Klebsiella pneumoniae* contribute to host cell association. *PLoS ONE*, **3**, e3817.
20 Cortés, G., Borrell, N., de Astorza, B., Gómez, C., Sauleda, J., and Albertí, S. (2002) Molecular analysis of the contribution of the capsular polysaccharide and the lipopolysaccharide O side chain to the virulence of *Klebsiella pneumoniae* in a murine model of pneumonia. *Infect. Immun.*, **70**, 2583–2590.
21 Cortés, G., de Astorza, B., Benedí, V.J., and Albertí, S. (2002) Role of the htrA gene in *Klebsiella pneumoniae* virulence. *Infect. Immun.*, **70**, 4772–4776.
22 Benghezal, M., Fauvarque, M., Tournebize, R., Froquet, R., Marchetti, A., Bergeret, E., *et al.* (2006) Specific host genes required for the killing of Klebsiella bacteria by phagocytes. *Cell. Microbiol.*, **8**, 139–148.
23 Maroncle, N., Rich, C., and Forestier, C. (2006) The role of *Klebsiella pneumoniae* urease in intestinal colonization and resistance to gastrointestinal stress. *Res. Microbiol.*, **157**, 184–193.
24 Glomski, I.J., Piris-Gimenez, A., Huerre, M., Mock, M., and Goossens, P.L. (2007) Primary involvement of pharynx and Peyer's patch in inhalational and intestinal anthrax. *PLoS Pathog.*, **3**, e76.
25 Francis, K.P., Yu, J., Bellinger-Kawahara, C., Joh, D., Hawkinson, M.J., Xiao, G., *et al.* (2001) Visualizing pneumococcal infections in the lungs of live mice using bioluminescent *Streptococcus pneumoniae* transformed

with a novel gram-positive lux transposon. *Infect. Immun.*, **69**, 3350–3358.
26 Doyle, T.C., Burns, S.M., and Contag, C.H. (2004) *In vivo* bioluminescence imaging for integrated studies of infection. *Cell. Microbiol.*, **6**, 303–317.
27 Harris, R.S., and Schuster, D.P. (2007) Visualizing lung function with positron emission tomography. *J. Appl. Physiol.*, **102**, 448–458.
28 Schuster, D.P., Kozlowski, J., and Hogue, L. (2003) Imaging lung inflammation in a murine model of Pseudomonas infection: a positron emission tomography study. *Exp. Lung Res.*, **29**, 45–57.
29 Beckmann, N., Tigani, B., Sugar, R., Jackson, A.D., Jones, G., Mazzoni, L., and Fozard, J.R. (2002) Noninvasive detection of endotoxin-induced mucus hypersecretion in rat lung by MRI. *Am. J. Physiol. Lung Cell. Mol. Physiol.*, **283**, L22–L30.
30 Tournebize, R., Doan, B., Dillies, M.A., Maurin, S., Beloeil, J., and Sansonetti, P.J. (2006) Magnetic resonance imaging of Klebsiella pneumoniae-induced pneumonia in mice. *Cell. Microbiol.*, **8**, 33–43.
31 Sheth, V., van Heeckeren, R.C., Wilson, A., Van Heeckeren, A., and Pagel, M. (2008) Monitoring infection and inflammation in murine models of cystic fibrosis with magnetic resonance imaging. *J. Magn. Reson. Imaging*, **28**, 527–532.
32 Judenhofer, M.S., Wehrl, H.F., Newport, D.F., Catana, C., Siegel, S.B., Becker, M., *et al.* (2008) Simultaneous PET-MRI: a new approach for functional and morphological imaging. *Nat. Med.*, **14**, 459–465.
33 Thiberville, L., Moreno-Swirc, S., Vercauteren, T., Peltier, E., Cavé, C., and Bourg Heckly, G. (2007) *In vivo* imaging of the bronchial wall microstructure using fibered confocal fluorescence microscopy. *Am. J. Respir. Crit. Care. Med.*, **175**, 22–31.
34 Cortez-Retamozo, V., Swirski, F.K., Waterman, P., Yuan, H., Figueiredo, J.L., Newton, A.P., Upadhyay, R., Vinegoni, C., Kohler, R., Blois, J., Smith, A., Nahrendorf, M., Josephson, L., Weissleder, R., and Pittet, M.J. (2008) Real-time assessment of inflammation and treatment response in a mouse model of allergic airway inflammation. *J. Clin. Invest.*, **118** (**12**), 4058–4066
35 Eckburg, P.B., Bik, E.M., Bernstein, C.N., Purdom, E., Dethlefsen, L., Sargent, M., *et al.* (2005) Diversity of the human intestinal microbial flora. *Science*, **308**, 1635–1638.
36 Anand, A.J., and Glatt, A.E. (1994) *Salmonella osteomyelitis* and arthritis in sickle cell disease. *Semin. Arthritis Rheum.*, **24**, 211–221.
37 Monack, D.M., Mueller, A., and Falkow, S. (2004) Persistent bacterial infections: the interface of the pathogen and the host immune system. *Nat. Rev. Microbiol.*, **2**, 747–765.
38 Phalipon, A., and Sansonetti, P.J. (2003) Shigellosis: innate mechanisms of inflammatory destruction of the intestinal epithelium, adaptive immune response, and vaccine development. *Crit. Rev. Immunol.*, **23**, 371–401.
39 Pizarro-Cerda, J., and Cossart, P. (2006) Bacterial adhesion and entry into host cells. *Cell*, **124**, 715–727.
40 Galan, J.E., and Wolf-Watz, H. (2006) Protein delivery into eukaryotic cells by type III secretion machines. *Nature*, **444**, 567–573.
41 Haraga, A., Ohlson, M.B., and Miller, S.I. (2008) Salmonellae interplay with host cells. *Nat. Rev. Microbiol.*, **6**, 53–66.
42 Hutchens, M., and Luker, G.D. (2007) Applications of bioluminescence imaging to the study of infectious diseases. *Cell. Microbiol.*, **9**, 2315–2322.
43 Contag, C.H., Contag, P.R., Mullins, J.I., Spilman, S.D., Stevenson, D.K., and Benaron, D.A. (1995) Photonic detection of bacterial pathogens in living hosts. *Mol. Microbiol.*, **18**, 593–603.
44 Wiles, S., Clare, S., Harker, J., Huett, A., Young, D., Dougan, G., and Frankel, G. (2004) Organ specificity,

colonization and clearance dynamics *in vivo* following oral challenges with the murine pathogen *Citrobacter rodentium*. *Cell. Microbiol.*, **6**, 963–972.

45 Sory, M.P., and Cornelis, G.R. (1994) Translocation of a hybrid YopE-adenylate cyclase from *Yersinia enterocolitica* into HeLa cells. *Mol. Microbiol.*, **14**, 583–594.

46 Charpentier, X., and Oswald, E. (2004) Identification of the secretion and translocation domain of the enteropathogenic and enterohemorrhagic Escherichia coli effector Cif, using TEM-1 beta-lactamase as a new fluorescence-based reporter. *J. Bacteriol.*, **186**, 5486–5495.

47 Mills, E., Baruch, K., Charpentier, X., Kobi, S., and Rosenshine, I. (2008) Real-time analysis of effector translocation by the type III secretion system of enteropathogenic *Escherichia coli*. *Cell Host Microbe*, **3**, 104–113.

48 Schlumberger, M.C., Muller, A.J., Ehrbar, K., Winnen, B., Duss, I., Stecher, B., and Hardt, W.D. (2005) Real-time imaging of type III secretion: Salmonella SipA injection into host cells. *Proc. Natl Acad. Sci. USA*, **102**, 12548–12553.

49 Enninga, J., Mounier, J., Sansonetti, P., and Tran Van Nhieu, G. (2005) Secretion of type III effectors into host cells in real time. *Nat. Methods*, **2**, 959–965.

50 Van Engelenburg, S.B., and Palmer, A.E. (2008) Quantification of real-time Salmonella effector type III secretion kinetics reveals differential secretion rates for SopE2 and SptP. *Chem. Biol.*, **15**, 619–628.

51 Drecktrah, D., Knodler, L.A., Howe, D., and Steele-Mortimer, O. (2007) Salmonella trafficking is defined by continuous dynamic interactions with the endolysosomal system. *Traffic*, **8**, 212–225.

52 Veiga, E., Guttman, J.A., Bonazzi, M., Boucrot, E., Toledo-Arana, A., Lin, A.E., *et al.* (2007) Invasive and adherent bacterial pathogens co-Opt host clathrin for infection. *Cell Host Microbe*, **2**, 340–351.

53 Zwart, W., Griekspoor, A., Kuijl, C., Marsman, M., van Rheenen, J., Janssen, H., *et al.* (2005) Spatial separation of HLA-DM/HLA-DR interactions within MIIC and phagosome-induced immune escape. *Immunity*, **22**, 221–233.

54 Kuijl, C., Savage, N.D., Marsman, M., Tuin, A.W., Janssen, L., Egan, D.A., *et al.* (2007) Intracellular bacterial growth is controlled by a kinase network around PKB/AKT1. *Nature*, **450**, 725–730.

55 Bower, J.M., Eto, D.S., and Mulvey, M.A. (2005) Covert operations of uropathogenic Escherichia coli within the urinary tract. *Traffic*, **6**, 18–31.

56 Mims, C., Dockrell, H.M., Goering, R.V., Roitt, I., Wakelin, D., and Zuckerman, M. (2004) Urinary tract infections, in *Medical Microbiology*, 3rd edn, Elsevier Mosby, London, pp. 241–249.

57 Ronald, A.R., and Nicolle, L.E. (2002) Infections of the upper urinary tract, in *Diseases of the Kidney and Urinary Tract*, 7th edn (ed. R.W. Schrier), Lippincott Williams and Wilkins, Philadelphia, pp. 941–969.

58 Srinivasan, U., Foxman, B., and Marrs, C.F. (2003) Identification of a gene encoding heat-resistant agglutinin in *Escherichia coli* as a putative virulence factor in urinary tract infection. *J. Clin. Microbiol.*, **41**, 285–289.

59 Lomberg, H., Jodal, U., Leffler, H., De Man, P., and Svanborg, C. (1992) Blood group non-secretors have an increased inflammatory response to urinary tract infection. *Scand. J. Infect. Dis.*, **24**, 77–83.

60 Svanborg, C., Frendeus, B., Godaly, G., Hang, L., Hedlund, M., and Wachtler, C. (2001) Toll-like receptor signaling and chemokine receptor expression influence the severity of urinary tract infection. *J. Infect. Dis.*, **183** (Suppl. 1), S61–S65.

61 Mulvey, M.A., Schilling, J.D., Martinez, J.J., and Hultgren, S.J. (2000) Bad bugs and beleaguered bladders: interplay between uropathogenic Escherichia coli

and innate host defenses. *Proc. Natl Acad. Sci. USA*, **97**, 8829–8835.
62 Wu, X.R., Sun, T.T., and Medina, J.J. (1996) *In vitro* binding of type 1-fimbriated Escherichia coli to uroplakins Ia and Ib: relation to urinary tract infections. *Proc. Natl Acad. Sci. USA*, **93**, 9630–9635.
63 Baorto, D.M., Gao, Z., Malaviya, R., Dustin, M.L., van der Merwe, A., Lublin, D.M. and Abraham, S.N. (1997) Survival of FimH-expressing enterobacteria in macrophages relies on glycolipid traffic. *Nature*, **389**, 636–639.
64 Duncan, M.J., Li, G., Shin, J.S., Carson, J.L., and Abraham, S.N. (2004) Bacterial penetration of bladder epithelium through lipid rafts. *J. Biol. Chem.*, **279**, 18944–18951.
65 Mulvey, M.A., Lopez-Boado, Y.S., Wilson, C.L., Roth, R., Parks, W.C., Heuser, J., and Hultgren, S.J. (1998) Induction and evasion of host defenses by type 1-piliated uropathogenic *Escherichia coli*. *Science*, **282**, 1494–1497.
66 Backhed, F., Soderhall, M., Ekman, P., Normark, S., and Richter-Dahlfors, A. (2001) Induction of innate immune responses by *Escherichia coli* and purified lipopolysaccharide correlate with organ- and cell-specific expression of Toll-like receptors within the human urinary tract. *Cell. Microbiol.*, **3**, 153–158.
67 Billips, B.K., Forrestal, S.G., Rycyk, M.T., Johnson, J.R., Klumpp, D.J., and Schaeffer, A.J. (2007) Modulation of host innate immune response in the bladder by uropathogenic *Escherichia coli*. *Infect. Immun.*, **75**, 5353–5360.
68 Wullt, B., Bergsten, G., Samuelsson, M., and Svanborg, C. (2002) The role of P fimbriae for *Escherichia coli* establishment and mucosal inflammation in the human urinary tract. *Int. J. Antimicrob. Agents*, **19**, 522–538.
69 Melican, K., Boekel, J., Mansson, L.E., Sandoval, R.M., Tanner, G.A., Kallskog, O., *et al.* (2008) Bacterial infection mediated mucosal signaling induces local renal ischemia as a defense against sepsis. *Cell. Microbiol.*, **10**, 1987–1998.
70 Walsh, P.C. (2002) *Campbell's Urology*, Saunders, Philadelphia.
71 Roberts, J.A. (1991) Etiology and pathophysiology of pyelonephritis. *Am. J. Kidney Dis.*, **17**, 1–9.
72 Palestro, C.J., Love, C., and Miller, T.T. (2007) Diagnostic imaging tests and microbial infections. *Cell. Microbiol.*, **9**, 2323–2333.
73 Kadurugamuwa, J.L., Modi, K., Yu, J., Francis, K.P., Purchio, T., and Contag, P.R. (2005) Noninvasive biophotonic imaging for monitoring of catheter-associated urinary tract infections and therapy in mice. *Infect. Immun.*, **73**, 3878–3887.
74 Jansen, A.M., Lockatell, C.V., Johnson, D.E., and Mobley, H.L. (2003) Visualization of *Proteus mirabilis* morphotypes in the urinary tract: the elongated swarmer cell is rarely observed in ascending urinary tract infection. *Infect. Immun.*, **71**, 3607–3613.
75 Justice, S.S., Hunstad, D.A., Seed, P.C., and Hultgren, S.J. (2006) Filamentation by *Escherichia coli* subverts innate defenses during urinary tract infection. *Proc. Natl Acad. Sci. USA*, **103**, 19884–19889.
76 Bullen, A. (2008) Microscopic imaging techniques for drug discovery. *Nat. Rev. Drug Discov.*, **7**, 54–67.
77 Helmchen, F., and Denk, W. (2005) Deep tissue two-photon microscopy. *Nat. Methods*, **2**, 932–940.
78 Mansson, L.E., Melican, K., Boekel, J., Sandoval, R.M., Hautefort, I., Tanner, G.A., *et al.* (2007). Real-time studies of the progression of bacterial infections and immediate tissue responses in live animals. *Cell. Microbiol.*, **9**, 413–424.
79 Ashworth, S.L., Sandoval, R.M., Tanner, G.A., and Molitoris, B.A. (2007) Two-photon microscopy: visualization of kidney dynamics. *Kidney Int.*, **72**, 416–421.
80 Molitoris, B.A., and Sandoval, R.M. (2005) Intravital multiphoton microscopy of dynamic renal processes. *Am. J. Physiol. Renal. Physiol.*, **288**, F1084–F1089.

81 Alencar, H., Mahmood, U., Kawano, Y., Hirata, T., and Weissleder, R. (2005) Novel multiwavelength microscopic scanner for mouse imaging. *Neoplasia*, **7**, 977–983.

82 Mansson, L.E., Melican, K., Molitoris, B.A., and Richter-Dahlfors, A. (2007b). Progression of bacterial infections studied in real time–novel perspectives provided by multiphoton microscopy. *Cell. Microbiol.*, **9**, 2334–2343.

83 Svoboda, K., and Yasuda, R. (2006) Principles of two-photon excitation microscopy and its applications to neuroscience. *Neuron*, **50**, 823–839.

84 Bacskai, B.J., and Hyman, B.T. (2002) Alzheimer's disease: what multiphoton microscopy teaches us. *Neuroscientist*, **8**, 386–390.

85 Yu, W., Sandoval, R.M., and Molitoris, B.A. (2007) Rapid determination of renal filtration function using an optical ratiometric imaging approach. *Am. J. Physiol. Renal. Physiol.*, **292**, F1873–F1880.

86 Russo, L.M., Sandoval, R.M., McKee, M., Osicka, T.M., Collins, A.B., Brown, D., *et al.* (2007) The normal kidney filters nephrotic levels of albumin retrieved by proximal tubule cells: retrieval is disrupted in nephrotic states. *Kidney Int.*, **71**, 504–513.

87 Lindquist, R.L., Shakhar, G., Dudziak, D., Wardemann, H., Eisenreich, T., Dustin, M.L., and Nussenzweig, M.C. (2004) Visualizing dendritic cell networks *in vivo*. *Nat. Immunol.*, **5**, 1243–1250.

88 Chieppa, M., Rescigno, M., Huang, A.Y., and Germain, R.N. (2006) Dynamic imaging of dendritic cell extension into the small bowel lumen in response to epithelial cell TLR engagement. *J. Exp. Med.*, **203**, 2841–2852.

89 Mairey, E., Genovesio, A., Donnadieu, E., Bernard, C., Jaubert, F., Pinard, E., *et al.* (2006) Cerebral microcirculation shear stress levels determine Neisseria meningitidis attachment sites along the blood-brain barrier. *J. Exp. Med.*, **203**, 1939–1950.

90 Drevets, D.A., and Bronze, M. (2008) Listeria monocytogenes: epidemiology, human disease, and mechanisms of brain invasion. *FEMS Immunol. Med. Microbiol.*, **53**, 151–165.

91 Dunay, I.R., Damatta, R.A., Fux, B., Presti, R., Greco, S., Colonna, M., and Sibley, L.D. (2008) Gr1(+) inflammatory monocytes are required for mucosal resistance to the pathogen *Toxoplasma gondii*. *Immunity*, **29**, 306–317.

92 Serbina, N.V., Jia, T., Hohl, T.M., and Pamer, E.G. (2008) Monocyte-mediated defense against microbial pathogens. *Annu. Rev. Immunol.*, **26**, 421–452.

93 Join-Lambert, O.F., Ezine, S., Le Monnier, A., Jaubert, F., Okabe, M., Berche, P., and Kayal, S. (2005) *Listeria monocytogenes*-infected bone marrow myeloid cells promote bacterial invasion of the central nervous system. *Cell. Microbiol.*, **7**, 167–180.

94 Drevets, D.A., Dillon, M.J., Schawang, J.S., Van Rooijen, N., Ehrchen, J., Sunderkötter, C., and Leenen, P.J. (2004) The Ly-6Chigh monocyte subpopulation transports *Listeria monocytogenes* into the brain during systemic infection of mice. *J. Immunol.*, **172**, 4418–4424.

95 Hell, S.W. (2007) Far-field optical nanoscopy. *Science*, **316**, 1153–1158.

96 Betzig, E., Patterson, G.H., Sougrat, R., Lindwasser, O.W., Olenych, S., Bonifacino, J.S., *et al.* (2006) Imaging intracellular fluorescent proteins at nanometer resolution. *Science*, **313**, 1642–1645.

97 Rust, M.J., Bates, M., and Zhuang, X. (2006) Sub-diffraction-limit imaging by stochastic optical reconstruction microscopy (STORM). *Nat. Methods*, **3**, 793–795.

98 Willig, K.I., Kellner, R.R., Medda, R., Hein, B., Jakobs, S., and Hell, S.W. (2006) Nanoscale resolution in GFP-based microscopy. *Nat. Methods*, **3**, 721–723.

99 Bertelsen, M. (2006) Multiplex analysis of inflammatory signaling pathways using a high-content imaging system. *Methods Enzymol.*, **414**, 348–363.

100 Wang, Y., Botvinick, E.L., Zhao, Y., Berns, M.W., Usami, S., Tsien, R.Y., and Chien, S. (2005) Visualizing the mechanical activation of Src. *Nature*, **434**, 1040–1045.

101 Wong, K.W., and Isberg, R.R. (2005) Yersinia pseudotuberculosis spatially controls activation and misregulation of host cell Rac1. *PLoS Pathog.*, **1**, e16.

102 Breart, B., Lemaitre, F., Celli, S., and Bousso, P. (2008) Two-photon imaging of intratumoral CD8+ T cell cytotoxic activity during adoptive T cell therapy in mice. *J. Clin. Invest.*, **118**, 1390–1397.

103 Kang, J.J., Toma, I., Sipos, A., McCulloch, F., and Peti-Peterdi, J. (2006) Quantitative imaging of basic functions in renal (patho)physiology. *Am. J. Physiol. Renal. Physiol.*, **291**, F495–502.

104 Molitoris, B.A., and Sandoval, R.M. (2006) Pharmacophotonics: utilizing multi-photon microscopy to quantify drug delivery and intracellular trafficking in the kidney. *Adv. Drug Deliv. Rev.*, **58**, 809–823.

12
The Issue of Species Specificity of Bacterial Infection, How to Address It Experimentally

Olivier Disson, Pascale Cossart, and Marc Lecuit

12.1
Introduction

An infectious disease results from the complex interplay between a pathogen and its host. In otherwise healthy individuals, pathogens responsible for invasive infections actively invade, proliferate and sometimes persist in the host, despite the existence of physical and immunological defenses against infection. These host microbial interactions result from a co-evolution of the pathogen and the host, and may have led to stringent specificity of microbes for their host, at the species, tissue and cell levels.

Specific interactions between a pathogen ligand and a receptor modulate the species permissiveness to a given microorganism by allowing or not microbial adhesion and/or entry.

Species specificity may also reflect differences in host responses to infection. Indeed, the responsiveness of the immune system is not uniform among species, even among mammals. For example, MyD88 plays a major role in innate immunity against pyogenic bacteria in mice, but a more restricted role in humans [1].

The understanding of the pathophysiology of a human infection as well as the *in vivo* testing of antimicrobial drugs and vaccines necessitate an animal model in which the infectious agent exhibits the same cell and tissue tropisms as in humans and manifests the same observable direct effects and indirect immunopathological damage as that which occurs in humans. Such a model should allow the *in vivo* relevance of results acquired using more reductionist *in vitro* or *ex vivo* approaches to be tested. Ideally, both the microbial pathogen and the animal model should be genetically amenable, thus allowing assessment of the role of critical microbial and host factors during the infectious process. The genetic manipulation of both the pathogen and the host might lead to a better understanding of their respective contributions in the complex interplay that results in disease.

Human pathogens are not necessarily human-specific pathogens. Indeed, most of them also infect non-human primates, even if the disease manifestations are often at variance with those observed in humans. Other relevant animal models

Bacterial Virulence: Basic Principles, Models and Global Approaches. Edited by Philippe Sansonetti
© 2010 WILEY-VCH Verlag GmbH & Co. KGaA, Weinheim
ISBN: 978-3-527-32326-5

can be defined by systematic screening of laboratory animals or by searching in the wild for species that are susceptible to the same pathogen or a related pathogen. Another strategy is the humanization of animals by human tissue grafting in immunocompromised recipient animals, such as SCID mice.

Host specificity can be viewed as an obstacle to studying infection in an animal model, but this species specificity can also be used as a tool to understand the molecular determinants which render the microorganism pathogenic for humans. Deciphering *in vitro* the ligand–receptor interactions between a pathogen and its host, enables the design and generation of transgenic or knock-in models in which the human receptor for the pathogen is expressed. The contribution of the ligand–receptor interaction to the pathophysiology of the infection can then be evidenced by the gain of function observed in the 'humanized' model.

12.2 Molecular Aspects of Species Specificity

Species specificity often relies on a specific interaction between a bacterial ligand and its receptor. Deciphering this ligand–receptor interaction *in vitro* is a key factor in the study of infection, both *in vitro* and *in vivo*. A number of host receptors have been successfully isolated by biochemical approaches (such as affinity chromatography or pull down) using a purified bacterial protein to identify a host cell receptor from a permissive cell extract. These approaches led to the identification of the following species-specific interactions: E-cadherin–Internalin (Ecad-InlA, *Listeria monocytogenes,* [2]) and the CbpA–hpIgR (*Streptococcus pneumoniae* [3]).

Another strategy is to screen a cDNA library from a permissive cell line to render a non-permissive cell line susceptible to adhesion/invasion of a pathogen or one of its putative ligands. The host receptors of Hepatitis C Virus (CD81, claudin-1 and occludin) were determined using such a strategy [4–6].

To validate the role of these interactions, non-permissive cells have to be transfected with a construct bearing the receptor cDNA in order to test the adhesion and invasion of the wild-type and isogenic microbial mutants for the putative ligand. A complementary approach is to invalidate the host receptor expression by use of RNA interference.

Cell specificity of interaction may result from the absence of receptor expression or its non-functionality, due to a post-transcriptional modification or a difference in the sequence between the permissive and non-permissive species.

To definitively validate species specificity *in vitro*, non-permissive cells can be transfected with vectors expressing the receptors from naturally permissive and non-permissive species, and whether the interaction between the bacteria and the transfected cells is indeed species-specific can then be determined.

Using these strategies, several species-specific host–pathogen interactions have been identified, such as the interactions between two invasion factors of *L. monocytogenes*, InlA and InlB, and their respective receptors Ecad and Met, as well as the interaction between the pilus of *Neisseria meningitidis* and CD46.

12.3
Modelization of Bacterial Invasion Using Human Tissues *Ex Vivo*

In vitro study of host–pathogen interactions is a reductionist approach enabling the identification of precise molecular interactions. However, understanding the pathophysiology of infection necessitates more complex systems where host target cells are connected to their environment which is composed of the extracellular matrix, interstitial fluids and neighboring cells of the non-immune as well as innate and adaptive immune systems and irrigated by the blood flow. Human tissue explants potentially constitute good alternative models to investigate host–pathogen interactions.

12.3.1
Ex Vivo Infection of Blood and Body Fluids

The most commonly used human tissue is the blood, which can help recapitulate the septicemic phase of an infection, characterize the immune response to blood-borne infections as well as the pathogen adaptation to this host compartment. Another studied body fluid is saliva, which is a major component of the innate and acquired immune defenses in the oropharynx. *Streptococcus pyogenes* (Group A streptococcus (GAS)) is a bacterium which commonly infects the human upper respiratory tract. Studies of GAS cultured *ex vivo* in saliva led to the determination of the critical role of several virulence factors necessary for survival and multiplication in saliva, such as Sic, SpeB and the two-component gene regulatory system SptR/S [7, 8]. These results are presented in more detail below.

12.3.2
Ex Vivo Infection of Solid Tissues

The human intestine defines a barrier between the host and the external environment. *Listeria monocytogenes*, *Salmonella typhimurium* or *Shigella flexneri* are enteroinvasive bacteria able to colonize and invade the intestinal and colonic tissues in humans. *S. flexneri* is responsible for acute rectocolitis in humans, its only natural host. Laboratory animals thus constitute poor models of shigellosis. In a recent report, Coron *et al.* [9] have characterized the early mucosal and neuronal lesions that occur following *S. flexneri* infection in human colonic explants obtained from patients undergoing surgical resection for colonic adenocarcinoma. Lesions observed with a wild-type strain of *S. flexneri* were attenuated when using a *sepA* mutant of *S. flexneri* which encodes for a serine protease involved in virulence. This proves the relevance of this model for further studies *ex vivo*.

The mammalian placenta is another complex tissue constituting the interface between the maternal blood and the fetus. Some pathogens, such as *L. monocytogenes*, responsible for materno-fetal infections, are able to cross it. As described in more detail in the dedicated paragraph, we have infected *ex vivo* human placental explants to study the mechanisms by which *L. monocytogenes* targets and crosses

the materno-fetal barrier, and revealed a role for the proteins InlA and InlB in this process [10].

12.4 *In Vivo* Modeling and the Quest for Relevant Animal Models

Mice are the most commonly used mammal models for studying human pathogenesis. They offer the advantages of relatively low cost, ease of manipulation, and wide availability of genetically-defined and genetically-modified animals. However, a number of human pathogens do not infect mice, and when an infection occurs, disease manifestations can be significantly different from the corresponding human disease. Studying infection processes in wild or laboratory animals other than mice may lead to the establishment of relevant animal models.

12.4.1 Non-human Primates

Due to their evolutionary closeness to the human species, non-human primates, and especially great apes, are often the best animal models that can be experimentally infected with human pathogens, as illustrated for HCV [11]. However, ethical and cost considerations greatly limit the use of this species. Other non-human primates have been used as models to study human pathologies. As early as 1911, Goldberger and Anderson showed that Rhesus macaques (*Macaca mulatta*) developed measles when inoculated with the filtered secretion from measles patients, demonstrating that the vector of this pathology is a virus. More recently, cynomolgus macaques (*Macaca fascicularis*) were shown to develop pharyngitis when inoculated with *S. pyogenes*, which enabled researchers to study the transcriptome of this bacterium *in vivo* during an infection [12]. Rhesus and cynomolgus macaques are the two most common non-human primate species in the world, but they still are large and costly animals. Large-scale experiments such as mutant screening cannot be undertaken in such animal models. The common marmoset (*Callithrix jacchus*) is a New World primate. Although less closely related to humans than Old World primates, this model can be attractive since it is smaller and has a shorter gestation period than macaques. In the infection field, it has mostly been studied as a model of infection for the HCV-related virus GBV-B. Recently, a team of researchers has reported the successful generation of transgenic marmosets with germline transmission of the transgene [13] paving the way for transgenesis projects in this species which are more closely related to humans than previous species used for transgenesis.

12.4.2 Non-primate Mammals

Precise observation of the pathologies that occur in the wild or screening of laboratory animals can also be used to identify an adequate model for the study of a

pathogen. *L. monocytogenes* is a bacterium that was reported by Pirie in 1927 to infect in the wild a species of gerbil (*Tatera lobegulae*) in South Africa. We have shown that a neighboring species, the Mongolian gerbil (*Meriones unguiculatus)* can be efficiently infected after oral inoculation which is the natural route of infection [14]. This model and others, are being used to study *L. monocytogenes* pathophysiology and are described in detail below.

Helicobacter pylori is a human pathogen which invades the gastric mucosa and plays a major role in the development of gastric adenocarcinoma and lymphoma in humans. The ferret was the first animal in which gastric ulceration was observed after the oral inoculation of a *H. pylori*-related bacterium, *H. mustelae*. Mongolian gerbil has since been described as the model of choice, since infection with *H. pylori* can result in the development of ulceration and gastric cancer, leading to a direct link between the infection and adenocarcinoma formation [15]. Components of the *H. pylori cag* island encode for a type-IV secretion system and induce epithelial responses *in vitro* that are linked to carcinogenesis. Compared to gerbils infected with wild-type *H. pylori*, gerbils colonized with *cag* island mutant strains develop significantly less severe gastritis [16], proving a role for the *cag* island in gastritis *in vivo*.

12.5
In Vivo Modeling with a Focus on the Pathogen rather than the Host

12.5.1
Studying a Closely-related Pathogen

Another strategy used to study human pathogens is to work with the nearest pathogen that induces a similar pathology in a genetically amenable model, such as mice, or even zebrafish.

A good example of such a strategy is the use of the parasite *Plasmodium berghei* in the mouse to model the interaction between *Plasmodium falciparum* and human. In the bacterial world, the infection of zebrafish *with Mycobacterium marinum* has been shown to reproduce the formation of granuloma, a classical hallmark of tuberculosis. These granulomas are organized aggregates of immune cells thought to be critical for restricting bacteria expansion. The unique transparency of this animal enabled the fate of the granuloma to be followed and led to the conclusion that they help disseminate the infection rather than containing it [17].

Salmonella enterica causes severe diseases in humans, ranging from gastroenteritis to systemic infections (typhoid fever). Systemic infections due to *Salmonella* serovars are species-specific: *S. enterica* serovar Typhi (*S. typhi*) specifically infects humans, whereas *S. enterica* serovar Typhimurium (*S. typhimurium*) infects mice, with some variations in the level of susceptibility between mouse genetic backgrounds. Since murine typhoid is similar to human typhoid in a number of ways, this animal model has been widely used to study typhoid fever [18]. Mice are however more resistant than humans to intestinal colonization by *Salmonella*. In order to model this infectious step, a streptomycin-pretreated mouse model of

enterocolitis has been developed which allows the host and bacterial factors contributing to intestinal immunopathology to be probed [19].

12.5.2
Modifying the Bacterium

Another method of circumventing the species specificity of a given pathogen is to modify it in order to adapt it to a new host. It is a routine technique used by virologist, notably for RNA viruses, given the substantial plasticity of their genomes. The procedure can be carried out by the passage of a pathogen in cells of the animal model to be used or directly *in vivo* in the host, as achieved by Marchetti *et al.* [20] who collected *H. pylori* from the feces of experimentally infected mice and use this bacterium to infect naïve animals, thereby enhancing the infectivity of the bacterium.

A less empiric approach is to build a bacterial strain with a modified ligand that renders it permissive for a new host. This strategy was followed by Wollert *et al.* who constructed a 'murinized' *Listeria* strain expressing a modified InlA protein enabled to interact with mouse Ecad, unlike wt InlA, that does not interact with mouse Ecad but only with human Ecad [21].

12.6
In Vivo Modeling: use of Human Xenografts to generate 'Humanized' Animal Models

For some of human-specific pathogens, modeling of human infections has been achieved by use of human–mouse chimeras, where severe combined immunodeficient (SCID) mice serve as recipients for transplants of human cells, tissues or even whole organs. These models enable the study of the host–pathogen interactions in an *in vivo* context [22].

SCID mice lack both T and B cells because of the lack of V(D)J DNA rearrangements involved in the generation of T and B antigen receptors. A few years after the characterization of this line by Bosma *et al.* [23], transplantation of human hematolymphoid cells into SCID mice (SCID-hu-PBL) enabled the various responses of the human adaptive immune system to be studied *in vivo* [24]. Notably, this model has been used to study infectious diseases, including HIV, which can infect human T cells within these chimeric mice [25], although this model is far from perfect for the study of AIDS pathogenesis.

Several tissue types of human adult and fetal origin, as well as cancer cells have also been successfully transplanted into SCID mice. Mercer *et al.* used such a model to study the replication of HCV *in vivo* [26]. Human hepatocytes were transplanted into SCID mice carrying a plasminogen activator transgene. This transgene targets urokinase over-production in the liver, resulting in a profoundly hypofibrinogenemic state and accelerated hepatocyte death. Transplanted hepatocytes, including human hepatocytes, exhibit a replicative advantage over the sur-

rounding murine hepatocytes. Chimeric liver containing both mouse and transplanted adult human hepatocytes were then generated. Replication of HCV was demonstrated to occur in the chimeric liver of this mouse. This model is to date the only small animal model in which HCV replication does occur.

Complete human organs from fetal tissue can also be engrafted into SCID mice. These organs obtain a vascular supply, even when placed in an ectopic location, grow, differentiate and reconstitute all their constituent cell types. Human intestinal xenografts implanted into the subscapular region of SCID mice for instance become vascularized with murine endothelial cells and develop a lumen constituted by human cells organized in the same manner as in the human intestine. These SCID-hu-INT mice have been used to study the interactions between enteroinvasive pathogens and human intestinal cells in an *in vivo* context.

As previously described, the pathological manifestations induced by *S. flexneri* are specific to the human species. It was demonstrated that after the direct inoculation of *S. flexneri* into the lumen of human intestinal xenografts in SCID-hu-INT mice, this bacterium could invade intestinal epithelial cells and produce extensive gut inflammation and colonic ulcerations that mimic the findings seen in human patients [27]. Depletion of neutrophils from SCID-hu-INT mice using the monoclonal antibody RB6-8C5 did not alter tissue damage in response to *Shigella* infection, while inflammation in the human intestinal xenograft was reduced. Most strikingly, there was a 20-fold increase in the number of intracellular bacteria in the human intestinal xenografts from neutrophil-depleted SCID-hu-INT mice, establishing the critical role of neutrophils in controlling the spread of *Shigella* in the human intestinal cells of SCID-hu-INT mice.

More recently, the use of the SCID-hu-INT mouse model to study *Shigella* infection enabled the demonstration of an *in vivo* mechanism of down-regulation of antimicrobial peptide gene expression upon *Shigella* infection [28].

Some limitations however restrict the use of these models. The murine environment may modify the properties of the transplanted cells. Transplanted fetal organs may also behave differently from fully mature adult organs. Regarding SCID-hu-INT, the transplanted intestines are free of flora and lack peristalsis. They also lack M-cells and Peyer's patches, which are critical targets for human enteropathogens. Variability of the xenograft from different donors may also alter the reproducibility of the results. Another possible limitation when these humanized models are used to study pathogen infections is that the SCID background precludes the study of immunity toward the pathogen and subsequent vaccination trials. This problem can however be overcome by simultaneous introduction of the human xenograft along with donor human PBLs, thymus and liver in order to reproduce the donor immune system in the SCID-hu mouse, an approach which is conceptually attractive, yet difficult to achieve on a large scale, as working with humanized-SCID mice is more costly and time consuming than working with classical mouse strains.

However, this technique led to significant progress in the study of certain pathogens such as *Shigella* [27], *H. pylori*, in a model of SCID mice with a stomach graft [29] and *S. pyogenes* in a model of a SCID mouse bearing a human skin xenograft [30].

12.7
Genetic Engineering to Circumvent Species-specificity and Generate 'Humanized' Animal Models for Human Infectious Diseases

12.7.1
Transgenesis

As previously described, species specificity limits the ability to study a host–pathogen interaction in a laboratory animal model. However, discovering the molecular determinant involved in host–pathogen interaction *in vitro* enables the generation of a 'humanized' animal model expressing the human receptor, which could render the animal model susceptible to the infection. This can be achieved by transgenesis or by a targeted knock-in genetic change, which is more physiological as it does not change the level and pattern of expression of the gene potentially involved in the species specificity.

These techniques can lead to two major advances if infection in the 'humanized' animal is achieved and comparable to that seen in the human, and if the bacterial mutant, which does not express the bacterial ligand of the host receptor, is attenuated in virulence in this model: (i) it highlights the role of the molecular interaction in the pathophysiology of the infection; (ii) it enables other virulence factors to be studied and drugs and vaccines to be tested, parameters which could not be analyzed in a non-permissive animal model.

The first model of a 'humanized' transgenic mouse engineered to circumvent species specificity was developed to study the role of the CD155, or human Poliovirus Receptor (hPVR), in the course of poliomyelitis [31]. The hPVR gene which is under the control of its promoter sequence was expressed in the tissues of these mice, and notably in the CNS. In contrast to infection of non-transgenic mice, in which the poliovirus does not replicate or cause paralysis, intracerebral inoculation of PVR transgenic mice with poliovirus resulted in viral replication in the brain and spinal cord and development of paralytic poliomyelitis similar to that observed in humans. However, inoculation of these transgenic mice with poliovirus via the oral route does not lead to poliomyelitis, even when hPVR is over-expressed on enterocytes and the poliovirus does attach to fragments of small intestine [32]. These results demonstrated that hPVR plays a major role in poliomyelitis once the virus is located in the brain, but alone is not sufficient to reproduce the entire infection process in this transgenic model. More recently, it has been reported that poliovirus may interact with the enterocytes and cross the intestinal barrier, when the viral inoculum is buffered with 3% $NaHCO_3$ and orally inoculated in a model of hPVR-tg mice lacking the beta/alpha interferon receptor (IFNAR) [33]. The infected hPVR-Tg/*Ifnar*KO mice all showed paralysis and all died while only 20% of hPVR-Tg mice died. These results suggest that the type 1 interferon response in mouse inhibits the proliferation of the poliovirus, while hPVR could be the only species-specific receptor involved.

We will present in the following sections transgenic models which have been engineered to express human gene products, and have enabled the host–bacterial

interactions of three human pathogens to be studied *in vivo*: the interaction between the *L. monocytogenes* InlA and its receptor Ecad in the course of gastroenteritis [34]; the role of the *N. meningitidis* pilus for the establishment of meningitis in a transgenic model expressing the human CD46 [35] and the contribution of the interaction between the *S. pyogenes* streptokinase and the human plasminogen in skin infections [36].

12.7.2 Knock-in

Expression of a transgene *in vivo* in a mouse model may however differ in terms of level and location in relation to the endogenous expression of the gene to be studied. A knock-in transgenic mouse line in which the endogenous gene or a part of it, is replaced by the human ortholog, represents a very attractive model to study the contribution of a gene to the course of infection. This was the case in the knock-in mouse line bearing a 'humanized' rather than mouse Ecad to study feto-placental listeriosis [14].

12.8 *Listeria* as a Model of Intestinal and Materno-Fetal Infections

Listeria monocytogenes (Lm), the causative agent of listeriosis, is a Gram-positive food-borne pathogen, which is able to cross the intestinal (IB), materno-fetal (MFB) and blood–brain barrier (BBB), thus leading to gastroenteritis, materno-fetal infections and central nervous system infections (meningitis and encephalitis) in humans.

Lm is a facultative intracellular bacterium which has the remarkable capacity to enter, survive and proliferate not only in phagocytic but also in non-phagocytic cells. This latter property has been studied extensively in cultured cells and is considered to be central to the pathophysiology of listeriosis. Several factors of *Lm* involved in the key steps of the infection cycle have been described. Two bacterial proteins mediate entry: InlA (also referred to as internalin) and InlB, both being sufficient for entry of coated beads into human epithelial cells, through their interaction with their respective receptors Ecad and Met. *Lm* then escapes the endocytic vacuole through the action of the listeriolysin (LLO), together with two phospholipases (PlcA and PlcB). *Lm* is then able to propel itself due to the action of ActA which mediates the actin-based intracytoplasmic movement and spread from cell to cell [37].

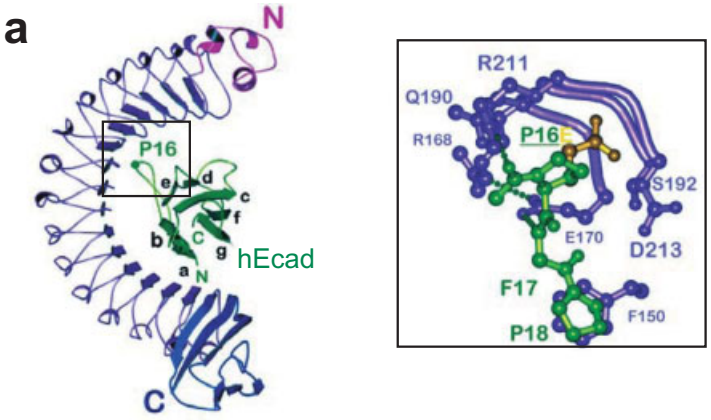
Lm was first isolated in 1924 by E.G.D. Murray in laboratory animals, namely rabbit and guinea pig. Almost simultaneously, Pirie described infections by *Lm* in South African gerbils [38] in the wild. The first cases of listeriosis in humans were recorded in 1929 in Denmark. *Lm* has also been reported to infect ruminants, especially sheep, in which CNS infection leads to 'circling disease', due to unilateral cranial nerve paralysis [39].

Until recently, mouse has been the most useful model for studying adaptive as well as innate immunity following *Lm* intravenous infection [40]. Models of infection in an invertebrate, *Drosophila melanogaster* [41] and in a fish species, *Danio rerio* [41a], have also been recently developed to study features of innate immunity in models that are easy to genetically engineer and image. In all these animal models, Δ*actA* and Δ*llo* null mutants are decreased in virulence after iv inoculation, highlighting the critical role of the intracellular lifestyle of *Lm in vivo*.

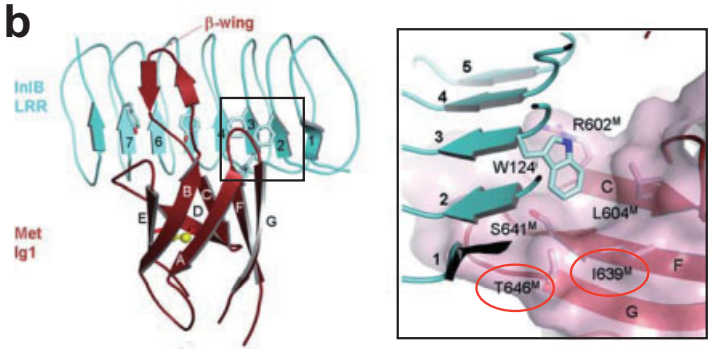
12.8.1 InlA and InlB are Involved in *Lm* Invasion of Non-phagocytic Cells *In Vitro* in a Species-Specific Manner

InlA has been shown to mediate internalization of *Lm in vitro* in human epithelial cells. The InlA receptor has been determined by a chromatography approach using a human epithelial cell extract, to be E-cadherin (Ecad), which is a component of the adherens junctions in epithelial cells [2]. Surprisingly, InlA has been shown not to induce *Lm* entry in epithelial cells expressing mouse or rat Ecad *in vitro*. We showed that the InlA–Ecad interaction is indeed species specific and relies on the sixteenth aa of the EC1 domain of Ecad, which is a proline in permissive species (e.g. human, guinea pig, rabbit) and a glutamic acid in non-permissive species (mouse and rat) [42]. These observations have been correlated with the structure of InlA in complex with the EC1 domain of Ecad [43]. The presence of the proline in position 16 allows the terminal loop of Ecad to be hydrophobic and uncharged, therefore strengthening the interaction with InlA. Replacing proline 16 with glutamic acid *in silico* in the EC1 domain of Ecad changes the hydrophilic properties of the binding site for InlA, therefore impeding a close interaction between receptor and ligand (Figure 12.1a).

Figure 12.1 Structural basis of the interaction between the invasion proteins InlA and InlB of *L. monocytogenes* and their respective species-specific receptors E-cadherin and Met. (a) Upper panel: structure of the human E-cadherin in complex with the Listeria invasion protein InlA. The species specificity of the InlA–E-cadherin interaction relies on the presence of a proline in position 16 of the N-terminal E-cadherin repeat, which allows the terminal loop of the E-cadherin to be hydrophobic and uncharged (adapted from [43, 44]). Lower panel: multiple alignment of the EC1 domain of Ecad. An asterisk '*' indicates identity throughout the column, a colon ':' a high conservation and a point '.' a weak conservation. Species are indicated on the left, the sixteenth amino acid position is a proline in permissive species (in red) and a glutamic acid in the non-permissive species (in red). (b) Upper panel: structure of the human receptor tyrosine kinase Met in complex with the Listeria invasion protein InlB. An enlargement of the hydrophobic pocket interacting with the tryptophan 124 of InlB is shown (from [47]). Lower panel: multiple alignment of Met domain interacting with InlB. An asterisk '*' indicates identity throughout the column, a colon ':' a high conservation and a point '.' a weak conservation. The horizontal line highlights the amino acid domain shown to interact with InlB. Species are indicated on the left, differences in amino acid sequences that may account for the non-recognition of rabbit and guinea pig Met by InlB are shown in green for the permissive interactions and in red for the putative non-permissive interactions.




```
Mouse          DWVIPPISCPENEKGEFPKNLVQIKSNRDKETKVFYSITGQGAD
Rat            DWVIPPINCPENQKGEFPQRLVQIKSNRDKETTVFYSITGPGAD
Gerbil         DWVIPPISCPENEKGPFPKDLVQIKSNRDKETKVFYSITGQGAD
Human          DWVIPPISCPENEKGPFPKNLVQIKSNKDKEGKVFYSITGQGAD
Rabbit         DWVIPPISCPENEKGPFPKNLVQIKSNRDKETQVFYSITGQGAD
Guinea pig     DWVIPPISCSENEKGPFPKRLVQIKSNRDKETKVFYSITGQGAD
               ******* *.**:** **: *******:***  ******* ***
                       |         |         |         |
                      10        20        30        40
```




```
Mouse          GPAMSEHFNVSVIISNSRETTQYSAFSYVDPVITSISPRYGPQAG
Rat            GPAMSEHFNVSVIVSNSRETTQYSAFSYVDPVITSISPRYGPHAG
Gerbil         GPAMSEHFNISVIISNSRETTQHSAFSYVNPVITGISPSYGPQAG
Human          GPAMNKHFNMSIIISNGHGTTQYSTFSYVDPVITSISPKYGPMAG
Rabbit         GATTHEHFNMSITVSNSRGRTQYSTFSYVAPVITSISPSYGPKAG
Guinea pig     GPSVNEHFNMSILVSNNRG-TAHSTFSYVDPVITSISPNYGPKAG
               *.:  :***:*: :**.:  * :*:**** ****.*** *** **
                  |         |         |         |         |
                 630       640       650       660       670
```

InlB, through its interaction with Met, also referred to as the Hepatocyte Growth Factor receptor, is also able to induce the entry of the bacterium into non-phagocytic cells *in vitro* [45]. We have investigated the InlB interaction with its receptor Met in several species. Using rabbit and guinea pig epithelial cell lines we were able to show that InlB does not interact with Met in these species, and is therefore unable to mediate entry *in vitro* [46]. The InlB–Met interaction is thus also species specific: it occurs in human, rat and mouse, but not in guinea pig and rabbit. The crystal structure of the complex between Met and the Met-binding domain of InlB has been defined [47]. By a sequence alignment of Met from permissive and non-permissive species, a difference in amino acids located in a hydrophobic pocket interacting with a tryptophan of InlB was demonstrated (Figure 12.1b). Experiments are now under way to assess the contribution of these amino acids to the species specificity of the InlB–Met interaction.

12.8.2 Gastroenteritis

The human gut defines a barrier between the external environment and the host. The lumen of the gut is the site of maintenance and proliferation of numerous commensal bacteria. Under some conditions this barrier may be invaded and crossed by certain pathogens, including bacteria such as *S. typhimurium*, *S. flexneri*, *Yersinia pseudotuberculosis* or *L. monocytogenes*. The gut barrier, at the small intestinal and colon level, is mainly composed of enterocytes – polarized epithelial cells harboring adherens and tight junctions – and also contains goblet cells, which produce the physically-protecting mucus, and M cells, which develop in contact with underlying B lymphocytes. Dendritic cells have also been shown to be able to sample the luminal content by extending pseudopods between epithelial cells. All these cells may constitute entry points for the bacteria to cross the intestinal barrier. The bacteria may reach the bloodstream directly or indirectly, and/or enter cells involved in the immune response (dendritic cells, monocytes, macrophages, neutrophils etc.), which could disseminate the bacteria throughout the whole organism.

In contrast to intravenous (iv) injection of *Lm*, oral inoculation in mouse is a very inefficient way to trigger a systemic listeriosis: *Lm* translocation across the intestinal barrier is very low and similar to that recorded for the non-pathogenic species *Listeria innocua*. This phenotype led to the hypothesis that InlA–Ecad interaction, which does not occur in mice, may play a role in the crossing of the intestinal barrier in permissive animal species.

In order to test this hypothesis, oral inoculations have been given to the guinea pig, a small animal model in which the InlA–Ecad interaction does occur. *Lm* was shown to target and cross the intestinal barrier in a InlA-dependent manner, inducing gastroenteritis and gaining access to the bloodstream (Figure 12.2a). To specifically study the role of the InlA–Ecad interaction in the crossing of the intestinal barrier, a transgenic mouse model was engineered. The human Ecad was expressed under the control of the promoter of the intestinal fatty-acid-binding

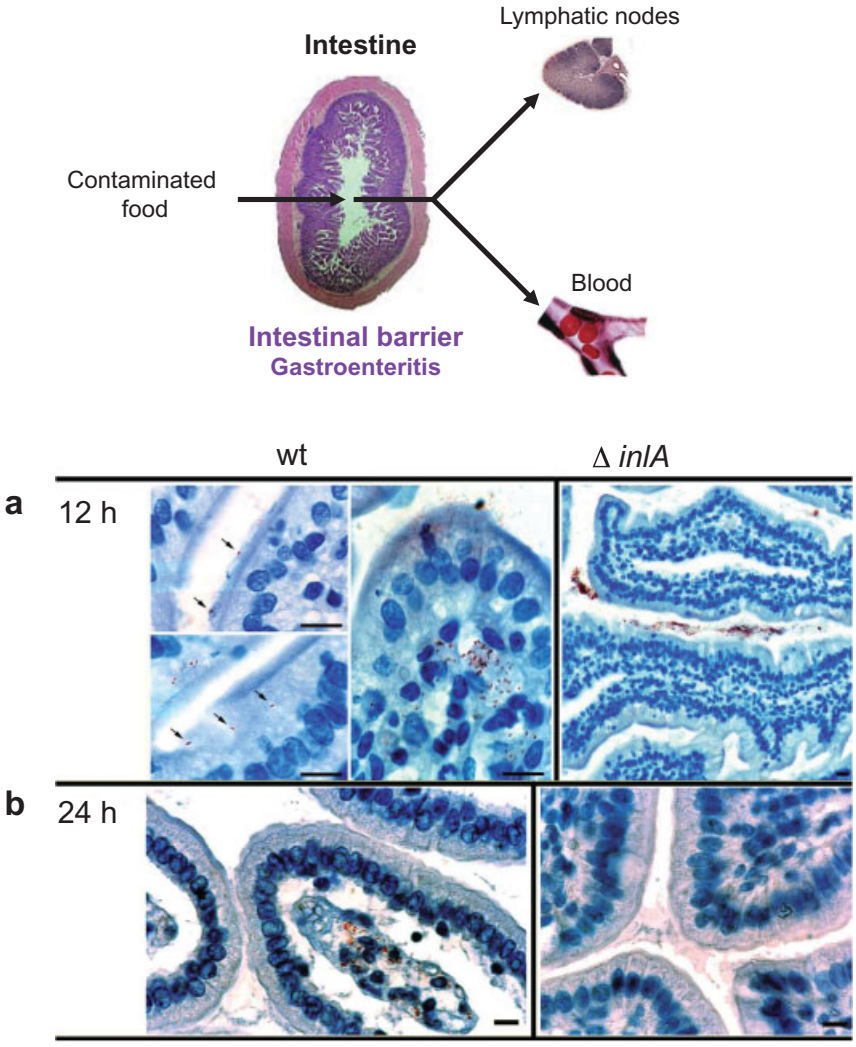


Figure 12.2 Role of InlA in the crossing of the intestinal barrier by *L. monocytogenes*. (a) Immunolabeling of wt *L. monocytogenes* or Δ*inlA* isogenic mutant (in red) invading the lamina propria after 12 h of infection in guinea pig. Scale bar: 10 µm. From [34]. (b) Immunolabeling of wt *L. monocytogenes* or Δ*inlA* isogenic mutant (in red) invading the lamina propria after 24 h of infection in E-cadherin transgenic mice. Scale bar: 10 µm. From [34].

protein (iFABP) gene, which is exclusively active in post-mitotic non-proliferative small intestinal enterocytes. In this model, orally inoculated *Lm* targets the enterocytes by interacting with the enterocytic Ecad. This interaction enables the crossing of the intestinal barrier (Figure 12.2b) and the downstream invasion of mesenteric lymph nodes, spleen and liver. This transgenic model was the first to

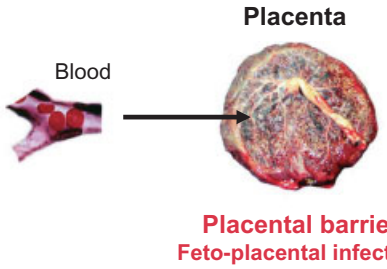
be used to determine the role of a species-specific bacterial virulence factor during the course of infection [34]. Specific expression of Ecad in enterocytes was necessary to demonstrate that this protein played a role in listeriosis by acting at the intestinal level, but the contribution of Ecad in other cell types in the gut or in other organs cannot be determined using such a model. To study the role of Ecad during all the steps of listeriosis *in vivo* in a mouse model, a knock-in (KI) E16P mouse strain, in which the glutamic acid at position 16 of the endogenous mouse Ecad is replaced by a proline, was designed. This amino acid substitution was previously shown to render the mouse Ecad permissive for interaction with InlA *in vitro* [42]. It was shown in this model that the InlA–E16PmEcad mediates the crossing not only of the small intestinal barrier, but also of the colon and cecum [14]. Of note, InlB does not play a role in the crossing of the gut barrier in transgenic or knock-in mice or in a guinea pig model [14, 46].

12.8.3 Materno-Fetal Infections

Several pathogens target the placenta (e.g. *Plasmodium falciparum*, [48]) or infect neonates during parturition (e.g. *Streptococcus agalactiae* or chikungunya virus [49]). Few pathogens, however, are able to infect the fetus during the pregnancy. This is true for *Lm* [50], the intracellular protozoan parasite *Toxoplasma gondii* [51], cytomegalovirus and the rubivirus [52, 53]. Invasion of the fetus may require a specific process of recognition and traversal of the feto-placental barrier which is composed of syncytiotrophoblasts.

Using trophoblast cells *in vitro* and human placental explants *ex vivo* (Figure 12.3a), we showed that in addition to InlB, InlA is also involved in the crossing of the feto-placental barrier [10]. However, *in vivo* analysis in guinea pig did not show a role for InlA, despite the permissiveness of this species toward the InlA–Ecad interaction [54]. In addition, InlB was shown to play no role in the traversal of the feto-placental barrier in mice [55]. These apparent contradictory results led us to formulate the hypothesis that both the InlA and InlB pathways have to be permissive *in vivo* in order to contribute to the crossing of the feto-placental barrier.

As previously mentioned, gerbils were shown to be susceptible to *Listeria* infection. This small rodent species harbors an Ecad with a proline in the sixteenth position, like all species that are permissive for the InlA pathway. Invasion assays of primary cells led to the conclusion that gerbil was indeed permissive for both InlA and InlB pathways [14]. In subsequent experiments, it was shown that both Δ*inlA* and Δ*inlB* mutants were much less efficient than wild-type bacteria in invading the placenta and fetus in gerbils after infection via the oral or intravenous route (Figures 12.3b and 12.4). To assess the causal relation between permissiveness to InlA and InlB pathways and InlA- and InlB-dependent placental invasion and fetal dissemination, a gain-of-function approach has been followed in the humanized E16P knock-in mouse line. In accordance with the results obtained in *ex vivo* explants and *in vivo* in gerbils, both InlA and InlB pathways were shown to act interdependently in the crossing of the feto-placental barrier in the KI E16P



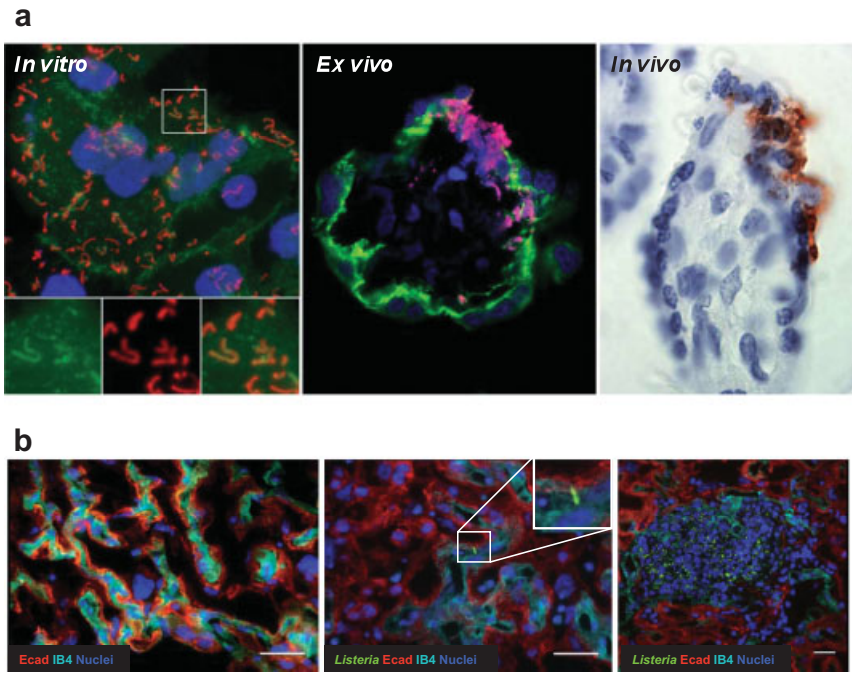
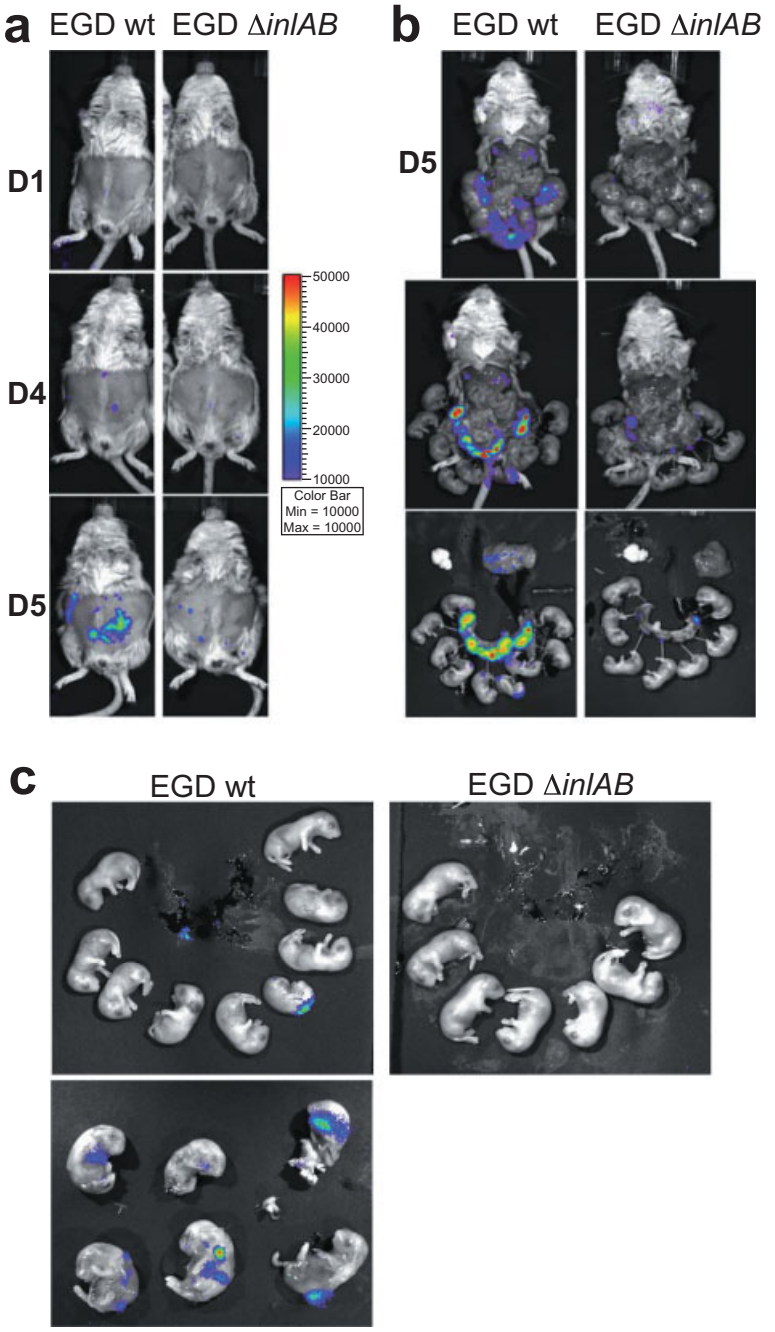


Figure 12.3 Traversal of the placental barrier by *L. monocytogenes* in human and gerbil. (a) Feto-placental listeriosis in human. Left: adhesion and invasion of bacteria expressing internalin in cultured human syncytiotrophoblast. Bacteria are shown in red, E-cadherin in green, and nuclei in blue. There is a recruitment of E-cadherin to the site of entry of the bacteria in the syncytiotrophoblast. Middle: section of a placental villus infected *ex vivo*. *L. monocytogenes* has invaded the syncytiotrophoblast and crossed the placental barrier. This has led to the constitution of villous microabscesses similar to those observed within placentas obtained from pregnant women with listeriosis (right). Bacteria are shown in purple in the middle image and in red in the right image. Adapted from [10]. (b) Feto-placental listeriosis in gerbil. Left: expression of E-cadherin (red) by the syncytiotrophoblast barrier which is bathed in maternal blood and surrounded by fetal capillaries (isolectin B4, cyan). Middle and right: sections of placentas 24 and 72 h post iv infection with the wild-type *L. monocytogenes*. In the middle image a bacterium located at the syncytiotrophoblast–feto-placental capillary interface is shown. In the right image, a placental abscess 72 h post-injection with the wild-type *L. monocytogenes* is shown. Scale bar: 10 µm. Adapted from [14].


a
EGD wt
EGD ΔinlAB
D1
D4
D5
50000
40000
30000
20000
10000
Color Bar
Min = 10000
Max = 10000
b
EGD wt
EGD ΔinlAB
D5
c
EGD wt
EGD ΔinlAB

mice. This result showed that the humanization of the Ecad not only enabled the InlA–Ecad interaction to occur at the feto-placental barrier, but also unmasked the role of the InlB–Met interaction in this phenotype. Taken together, this was the first report to demonstrate a specific recognition of the placental barrier by a microbial pathogen [14]. It is notable that this mechanism is different from those occurring at the intestinal level, where the InlA–Ecad interaction, but not the InlB–Met is involved in the traversal of the barrier. Further investigations are underway using these newly described animal models to understand what makes the mechanisms of traversal of these two epithelial barriers different (Figure 12.5).

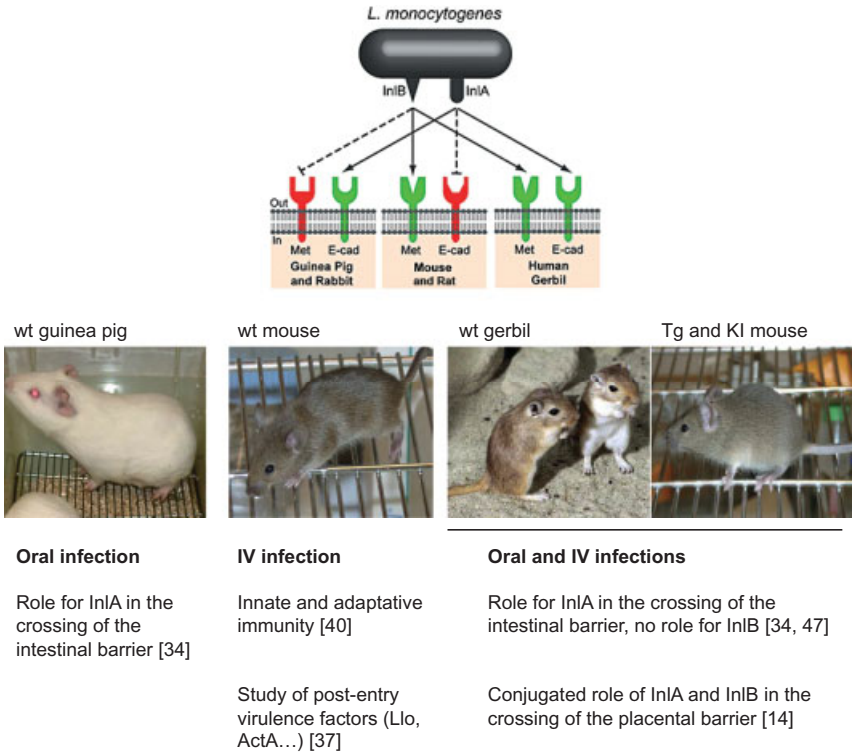


Figure 12.5 Animal models used for the study of human listeriosis.

Figure 12.4 *inlAB*-dependent invasion of placenta and fetuses in pregnant gerbil infected via the intravenous route. (a) Time course of infection of pregnant gerbils inoculated intravenously with 2×10^6 bioluminescent EGD WT *L. monocytogenes* or EGD Δ*inlAB* mutant. (b) Dissection of the same animals on day 5 after infection, showing a far greater placental bioluminescence with EGD WT than Δ*inlAB*. (c) Fetuses retrieved from two EGD WT or one EGD Δ*inlAB*-inoculated pregnant gerbil on day 5 after infection. Adapted from [14].

12.9
Streptococcus pyogenes Infections: from Colonization of Host Epithelial Barriers to Systemic Infection

12.9.1
Streptococcus pyogenes (GAS) as a Model of Pharyngitis and Skin Infections

Streptococcus pyogenes, or Group A streptococcus (GAS) is the most common cause of bacterial pharyngeal and skin infections. This extracellular Gram-positive bacterium is highly host-specific and infects only humans. GAS also exhibits a tissue tropism, since the bacterial strains recovered from the throat are of different serotypes from those recovered from skin infections. GAS is able to induce severe life-threatening invasive infections such as necrotizing fasciitis, streptococcal toxic shock and myositis. Colonization of the oropharynx is also associated with rheumatic fever, an autoimmune disorder which is the leading cause of preventable childhood heart disease in the developing world [56].

Because of the strict restriction of GAS to humans, *in vivo* models of infection in mice are not pertinent to the study of this pathogen. *In vitro* experiments have shown a highly specific degradation of human plasminogen by the streptokinase, a GAS-secreted enzyme [57], which could be involved in the invasion phenotype of GAS. This discovery led to the implementation of a transgenic mouse model expressing human plasminogen [36]. *Ex vivo* experiments in human saliva as well as analysis of the bacterial transcriptome in a model of pharyngitis in macaques or studies in human skin-grafted mice also led to the discovery of new mechanisms of infections (see also [58] for a review).

12.9.2
Skin Infections

The ability of GAS to cause invasive disease requires the invasion of host tissues. The proteolytic digestion of host proteins has been hypothesized as a mechanism by which the bacterium disrupts host barriers in order to penetrate the systemic compartment.

The plasminogen system is an attractive target for GAS in the invasion process. Plasmin is the major serine protease that degrades fibrin blood clots. This enzyme is generated through cleavage of the proenzyme plasminogen (PLG) by the host plasminogen activators, which are the tissue type plasminogen activator (tPA) and urokinase type plasminogen activator (uPA). It has been demonstrated *in vitro* that the GAS plasminogen-binding group A streptococcal M-like protein (PAM), a surface-associated bacterial factor, binds directly to the human plasminogen [59]. This interaction is species-specific and has been investigated: the lack of interaction between PAM and the murine plasminogen is due to two amino acid replacements in the plasminogen kringle-2 domain (K2, see Figure 12.6) which in humans binds to the VEK-30 internal peptide from PAM [61]. The human PLG can then be activated by the streptokinase (SK), a secreted GAS enzyme, which is also

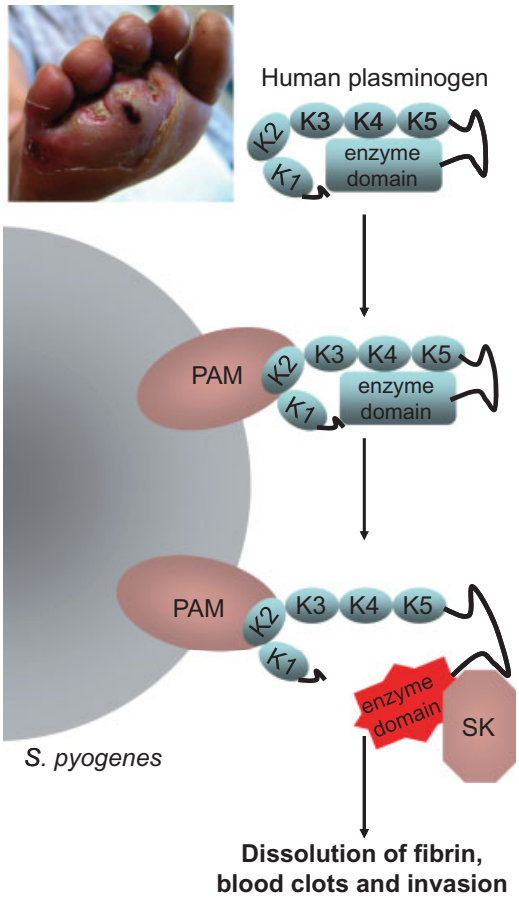


Figure 12.6 Plasminogen activation mechanisms used by *S. pyogenes* and pathophysiological consequences in human. Upper image: impetigo due to *S. pyogenes* in human. In the first step the human plasminogen is recruited to the bacterial surface by the interaction between the kringle domains 2 (K2) of the plasminogen and the protein PAM (Plasminogen-binding group A streptococcal M-like protein) from *S. pyogenes*. In the second step, the surface-bound plasminogen is activated by the streptokinase (SK) of *S. pyogenes*, thus allowing the bacteria to dissolve the fibrin blood clots. Adapted from [60].

unable to interact with PLG from species other than human [57] (Figure 12.6). Studies of streptococcal pathogenicity and the role of SK and surface-bound PLG-binding proteins *in vivo* have been hampered by this restricted host specificity.

In an attempt to study the contribution of SK and PAM to skin infection *in vivo*, Svensson *et al.* used the hu-skin-SCID mouse model in which human neonatal foreskins are grafted onto the backs of SCID mice. Applying GAS on the skin grafts resulted in an infection that closely mimics human impetigo [30]. Using this hu-skin-SCID mouse model in conjunction with isogenic mutants of GAS,

the authors showed that the secreted cysteine proteinase SpeB plays a key role in virulence, which is correlated to the tissue specificity of some GAS strains for skin infection versus throat infection [62]. In this hu-skin-SCID mouse model, inactivation of genes encoding either PAM or streptokinase led to a partial, but significant, loss of virulence *in vivo*, as measured by the net growth of the bacteria and pathological alterations. The relative loss in virulence *in vivo* was greater for the streptokinase mutant than for the PAM mutant. The combined experimental and epidemiological data provide evidence that PAM and streptokinase play a key role in mediating skin-specific infection by GAS, but the host factor was not strictly defined [63].

Another method of circumventing this host specificity was to inject human plasminogen into the mouse skin before infection with GAS [64]. The area of the skin lesion and mortality after bacterial inoculation were shown to be significantly greater with a SK-expressing strain than with the isogenic Δ*sk* mutant in the presence of human PLG. Human PLG also enhanced the virulence of the Δ*sk* mutant, suggesting that PLG interacts with other bacterial factors that contribute to the virulence (e.g. PAM and other potential factors) and could then be activated by host factors such as tPA or uPA. These results suggested that the SK–PLG interaction is a key step in species-specific GAS invasion *in vivo* and that both SK and host PLG activators appear to promote virulence of GAS by catalyzing plasmin formation.

Based on these findings, a 'humanized' transgenic mouse expressing human PLG under the control of the albumin gene regulatory sequence was generated in *plg* –/– mice [36]. Mouse mortality due to GAS skin infection was significantly enhanced in this transgenice mouse model (75% compared to 20% in littermates infected with a strain exhibiting enhanced virulence in the mouse skin infection model). The mortality in transgenic 'humanized' mice was decreased to 27% when infected with the isogenic SK mutant (*ska*), demonstrating the contribution of SK to host specificity. The potential role of PAM was then examined in this model. The increased mortality of GAS-infected transgenic mice was also abolished when the animals were inoculated with an isogenic PAM mutant. The authors hypothesized that GAS exploits the plasminogen system to circumvent local defenses, which would otherwise result in local thrombosis and microvascular occlusion, in order to gain access to the vascular system (Figure 12.6). In fact, virulence after GAS inoculation by the venous route was not modified in transgenic mice compared to controls, proving that the differences in virulence were due to the interaction of GAS with PLG at the skin level and not at the systemic level.

In a recent report [65], the authors confirmed the role of thrombin generation in antimicrobial host defense. Use of mice with a deficiency in factor V (FV, a critical component of the prothrombinase complex) resulted in markedly increased mortality after GAS infection, suggesting a role for the platelet FV pool in host defense against GAS. Mice with complete deficiency of fibrinogen also demonstrated markedly increased mortality to GAS infection relative to controls. These results support the hypothesis that local thrombosis/fibrin deposition limits the

survival and dissemination of at least a subset of microbial pathogens. Disruption of fibrin deposition by PLG activated by SK could then be a key event in GAS invasion *in vivo* after a skin invasion.

12.9.3 Models of Pharyngitis

12.9.3.1 *Ex Vivo*

Studying pharyngeal infection caused by GAS has long been hampered by the lack of pertinent models. Saliva plays a central role in GAS transmission and is an easy-to-handle tissue for the *ex vivo* study of expression of virulence factors which could be potentially induced during pharyngeal infections. Shelburne and co-workers investigated GAS–saliva interactions in order to study virulence factors that enable GAS to survive and proliferate in this environment which is a key mediator of innate and acquired immunity in the human oropharynx. Using isogenic mutants of GAS, they determined that two virulence factors, streptococcal inhibitor of complement (Sic) and streptococcal pyrogenic exotoxin B (SpeB), enable GAS to survive in saliva [8]. These authors then analyzed the transcriptome of a GAS strain grown in saliva [7], and characterized two new genes whose transcript is modulated in saliva, sptR and sptS (sptR/S) that encode a two-component gene regulatory system. SptR/S optimizes persistence of GAS in human saliva since an isogenic polar mutant strain (DeltasptR) was less able to survive in saliva compared with the parental strain, apparently by influencing metabolic pathways and virulence factor production.

12.9.3.2 *In Vivo*

The lack of an animal model that recapitulates the clinical manifestations of GAS pharyngitis in humans has been a major obstacle for understanding GAS pharyngeal pathogenesis. GAS has been shown to colonize the oropharynx of baboons, but similar to mice, these primates do not develop pharyngitis following GAS inoculation [66]. However, a human-like pharyngitis was shown to occur in cynomolgus macaques following experimental GAS inoculation [12]. Using real-time RT-PCR, analysis expression profiles of a subset of GAS genes was shown to reflect the profiles observed in human pediatric patients. In order to gain a broad picture of how bacterial gene expression changes over time during experimental pharyngitis in cynomolgus macaques, the transcriptome of group A Streptococcus (GAS) was analyzed using a 3-month infection protocol [67]. The temporal pattern of GAS gene expression in PHG was shown to be linked to three distinct phases of infection: colonization, inflammation and an asymptomatic carriage phase. Successful colonization and severe inflammation were significantly correlated with an early onset of superantigen gene expression, which may explain the elevated immune response induced by the infection. The expression of the two-component regulator *covR* was up-regulated in the latest phase of disease, when CFUs were decreasing, while another two-component regulator, *spy0680* was significantly up-regulated during the colonization phase. It has now to be

demonstrated that these two-component regulators play a role in establishing or prolonging GAS–host interactions in humans. This study is a starting point for the targeted investigation of proven and putative virulence factors and genes of unknown function which may be involved in pharyngitis.

12.9.4 CbpA, the Adhesin of *Streptococcus pneumoniae* Involved in Species Specificity for Humans

Streptococcus pneumoniae or pneumococcus, is a major pathogen that causes invasive infections such as sepsis, pneumonia, and meningitis in humans. Pneumococci colonize the nosopharyngeal epithelium and may cross the nasopharynx or lung epithelial barrier to reach the bloodstream. The polymeric immunoglobulin receptor (pIgR) is a membrane protein which transports the polymeric imunoglobulins across the mucosal epithelium. Zhang *et al.* have determined that a major pneumococcal adhesin, CbpA (also referred to as PspC, SpsA and PbcA), may interact with pIgR expressed by human nasopharyngeal cells *in vitro* [3]. Expression of human pIgR (hpIgR) in non-permissive MDCK cells greatly enhances pneumococcal adherence and invasion. The hpIgR-mediated bacterial adherence and invasion are abolished by mutation of *cbpA* or antibodies against hpIgR. In contrast, rabbit pIgR (rpIgR) does not bind to CbpA and its expression in MDCK cells does not enhance pneumococcal adherence and invasion. Finally, using *cbpA* mutants, these authors have shown that interaction of CbpA with hpIgR mediates transmigration of the pneumococci across an *in vitro* barrier of human nasopharyngeal epithelial cells. In a subsequent report [68], the submolecular characterization of this interaction was defined. Whereas the mouse pIgR does not bind CbpA, a chimeric mouse pIgR bearing the third and fourth Ig-like domain of human pIgR was proven to bind CbpA, both in an enzymatic assay or after the transfection of non-permissive cells with this construction. These results suggest that pneumococci may use the pIg transcytosis machinery to promote translocation across the mucosal barrier in a species-specific manner. However, the *in vivo* net contribution of human pIgR in pneumococcus infection remains to be investigated, since mouse pIgR has also been shown to protect against nasopharyngeal carriage of *S. pneumoniae in vivo* [69].

CbpA has also been shown to bind to the human complement factor H (FH), but not the mouse, rat, rabbit, horse or bovine FH [70]. FH is a complement alternative pathway inhibitor and its recruitment by *S. pneumoniae* may thus constitute an immune evasion mechanism against complement-mediated bacterial clearance in humans.

CbpA may thus play a role during different stages of infection in humans, for traversal of the mucosal barrier and survival in the bloodstream. However, an animal model which recapitulates the pathologies induced by *S. pneumoniae* remains to be developed. A transgenic mouse model co-expressing the human pIgR and/or the human FH would be very helpful in investigating this pathology *in vivo*.

12.10
Neisseria meningitidis and Cerebrospinal Meningitis

12.10.1
Neisseria meningitidis

The blood–brain barrier (BBB) separates the blood from the central nervous system. Bacteria such as *L. monocytogenes*, *S. pneumoniae* or *N. meningitidis*, are able to cross the BBB, suggesting that they possess specific virulence factors that interact with the BBB.

N. meningitidis is a commensal Gram-negative bacterium of the human nasopharynx which colonizes 3–30% of the population. Under unknown circumstances, this bacterium, which is specific to human, is able to cross the mucosal epithelium, resulting in bacteremia. Once in the bloodstream, it is capable of crossing the blood–brain barrier and causing meningitis.

The colonization of the human epithelium of the nasopharynx is essential for the persistence of this bacterium and its subsequent pathogenesis. Adhesion to and traversal of the blood–brain barrier is the second key step leading to the manifestation of meningitis. We will present here studies investigating the role of human cell surface glycoprotein CD46 in pilus-mediated adhesion of *N. meningitidis*, which may play a role in several of the steps in *Neisseria* invasion, although its precise role in the crossing of the BBB remains controversial (see also [71] for a review). Other molecular interactions that could explain the tropism for the human epithelium and brain endothelium will also be presented.

12.10.2
CD46 and Meningitis

CD46, or membrane cofactor protein (MCP), is a transmembrane glycoprotein which has been identified as a receptor for several human viruses (measles virus [72], human herpesvirus 6 [73], and adenoviruses of different serotypes[74]) and the bacterium *S. pyogenes* [75].

CD46 was identified by Källström *et al.* as a cellular receptor for the pilus of *N. meningitidis*, as well as *Neisseria gonorrhea*, the causative agent of gonorrhea [76]. Purified pili were found to bind to CD46 on SDS-PAGE of extracts from ME-180 human cervical epithelial cells.

Piliated *N. meningitidis* and *N. gonorrhea*, but not non-piliated bacteria, were then shown to adhere to Chinese hamster ovary (CHO) cells transfected with human CD46, but not to untransfected cells. Both pilus components PilE (the major pilus subunit) and PilC (a pilus-associated protein that influences pilus fiber assembly) may interact with CD46. CD46 then became an attractive candidate as a pilus receptor since its expression has been demonstrated to occur in tissues that are relevant to *Neisseria* infection, including blood vessel endothelial cells of the brain.

Based on this finding, Johansson *et al*. investigated the role of CD46 in transgenic mice that expressed human CD46 [35]. These mice, which exhibit a human-like tissue distribution of CD46 were shown to display enhanced susceptibility to meningococcal disease following experimental inoculation via either the intraperitoneal or intranasal route. Following intraperitoneal infection, bacteremia and mortality were higher in CD46 transgenic mice, which were also more susceptible to central nervous system infection, suggesting a role for CD46 in facilitating the crossing of the blood–brain barrier. But surprisingly, the non-piliated isogenic strain of *N. meningitidis* was more virulent than the piliated strain following intraperitoneal inoculation in mice expressing CD46, thus challenging the proposed role of CD46 as a pilus receptor in brain infection. Intranasal inoculation, which more closely mimics the natural route of infection, resulted in no mortality among non-transgenic animals and only 15% lethality among CD46-transgenic mice that were infected with the piliated strain. In contrast to the increased virulence that was observed following intraperitoneal inoculation, non-piliated meningococci were avirulent following an intranasal challenge in the transgenic animals, which is consistent with the proposed role of CD46 pilus interaction in the adhesion of bacteria to epithelial cells in the nasal cavity. However, the low mortality rate that was observed via this natural inoculation route and the unexpected virulence exhibited by non-piliated meningococci following intraperitoneal inoculation, emphasize the complexity of the role of CD46 in the progression of *Neisseria* disease. In this model the CD46–pilus interaction seems to play a role in the colonization of the nasopharynx. CD46 may also be involved in the traversal of the blood–brain barrier but in a pilus-independent manner. Further studies in CD46 transgenic mice [77] showed that bacterial clearance is delayed in CD46 transgenic mice, correlating with a higher level of expression of the pro-inflammatory cytokines TNF and IL-6, and the anti-inflammatory cytokine IL-10, similar to that observed in patients with lethal meningococcal disease. Basal level of C5a (generated upon activation of the complement cascade) was also shown to be higher in CD46 tg mice, independently of the infection. CD46 may then play a role in modulating the immune response induced by the pathogen leading to the establishment of severe bacteremia rather than acting as a receptor to facilitate the passage of *Neisseria* across the blood–brain barrier.

At present, the identity of CD46 as a cellular pilus receptor for *Neisseria* remains controversial. Two independent studies demonstrated that the highest levels of bacterial adherence did not correspond to the highest levels of CD46 expression [78, 79]. Most importantly, in contrast to other studies, no binding of piliated *N. gonorrhea* or PilC2 protein was observed on CD46-transfected CHO and MDCK cells and down-regulation of CD46 expression in human epithelial cell lines by RNA interference did not alter the binding efficiency of piliated gonococci or purified PilC2 protein. These data support the notion that pilus-mediated gonococcal infection of epithelial cells can occur in a CD46-independent manner, thus questioning the function of CD46 as an essential pilus receptor for pathogenic *Neisseria*.

12.10.3
Other Molecular Interactions at the Blood–Brain Barrier Level

Efforts to define the mechanism by which *N. meningitidis* targets and crosses the BBB recently led to new discoveries. It has first been shown that the type IV pilus of *N. meningitidis*, and especially the PilC protein which is responsible for the adhesion property of the pilus, plays a major role in the adhesion of *N. meningitidis* to a human brain endothelial cell line (hCMEC/D3) under shear stress that mimics the blood flow [80]. Additionally, a mechanism by which *N. meningitidis* could target and cross the BBB has recently been shown [81]. Using the *in vitro* model of hCMEC/D3 cell line, the authors claimed that the type IV pilus recruits the Par3/Par6/PKCζ polarity complex, thereby leading to the opening of the intercellular junctions. The bacteria may then cross the BBB by a paracellular route. These results have however to be confirmed in a pertinent animal model. A candidate for a *N. meningitidis* receptor at the BBB level has been recently proposed. It has indeed been shown that laminin receptors (LR) from mouse as well as from humans are recognized by Neisseria PilQ (a type IV pilus secretin protein) and PorA (major outer membrane protein) [82]. These interactions allow binding of beads bearing these bacterial proteins to the cerebral endothelial LR *in vivo* in mice. This interaction does not occur in animals treated with antibody against LR. Laminin receptors could then be one of the receptors that enable the adhesion of *Neisseria* to the blood–brain barrier but in a non-species-specific manner.

12.10.4
Other Host-specific Interactions which occur during Meningococcal Infection

Another element of host specificity is the system of meningococcal iron uptake by transferrin-binding proteins that specifically bind human transferrin but not mouse transferrin [83]. A mouse model for experimental meningococcal infection using transgenic mice expressing human transferrin has been developed [84]. Intraperitoneal challenge of transgenic mice induced bacteremia and inflammation in the meningeal subarachnoidal space, whereas the initial inoculum was rapidly cleared from the blood in wild-type mice. *N. meningitidis* mutants that were unable to use transferrin as a source of iron were rapidly cleared from both wild-type and transgenic mice. This transgenic mouse model expressing human transferrin is of particular interest and can now be crossed with other mouse strains, for example, human CD46 transgenic mice, in order to recapitulate the human disease *in vivo* and to study meningococcal virulence and immunogenicity factors in more detail.

12.11
Concluding Remarks

As illustrated in this chapter, species specificity constitutes a limitation to the study of the pathophysiology of infection, but at the same time also offers the

Table 12.1 *In vivo* species-specific interactions and relevant animal models.

Pathogen	Pathology	Permissive models	Species-specific interaction	Reference
Listeria monocytogenes	Gastroenteritis	Guinea pig, rabbit, gerbil	InlA–Ecad	[34]
		hEcad tg mice mEcad E16P KI mice	InlA–Ecad	
	Materno-fetal infection	gerbil	InlB–Met	[14]
		mEcad E16P KI mice	Pilus–CD46?	
Neisseria meningitidis	CNS infection	CD46 transgenic mice		[35]
Streptococcus pyogenes	Skin infection	Plasminogen tg mice	Streptokinase–plasminogen	[36]
	Pharyngitis	Cynomolgus macaque	ND	[12]
Helicobacter pylori	Gastric adenocarcinoma	Stomach grafted mice		[15, 29]
		Gerbil	ND	
Shigella flexneri	Gastroenteritis	Hu-SCID-Int		[27]

ND, not determined.

opportunity to assess the contribution of these interactions to the infection process in suitable animal models (table 12.1).

Species specificity also reflects the co-evolution of pathogens and their hosts and the equilibrium between these two partners in the infection process: the pathogen is not only one of the factors which drives the selection of resistant individuals, but it also takes advantage of the host permissiveness for its amplification and transmission among susceptible hosts. As an example, *Plasmodium falciparum*, the causative agent of malaria, has been shown to drive the selection of a single copy of the Hb allele of the β-globin gene, although it is detrimental when homozygous, causing sickle-cell disease [85]. At the individual level, pathogens also affect non-heritable traits [86], such as those encoding the effectors of adaptive immunity, as well as epigenetic modification that might occur during infection [87, 88].

The host in return also drives the selection of pathogens whose genomes are more plastic than that of the host. Once a first round of co-evolution is achieved, the cycle of the pathogen within its host is more or less fixed, enabling both pathogen and its dedicated host populations to be maintained over time. Whether the species specificity of some of the ligand–receptor interactions described in this chapter is a consequence of such a co-evolution, or only the result of the preferential recognition by pathogen ligands of their receptors which harbor a

pre-existing difference among the homologous genes in other species, remains to be investigated. As an example, it has been proposed that the specific interaction between the streptokinases (SK) from different streptococcus strains and plasminogen is the consequence of a co-evolution [89]: comparative sequence analysis indicates that the sites in plasminogen that interact with SK are preferentially targeted for mutation. Conversely, intermolecular contact sites in SKs that activate human plasminogen are more highly conserved than other loci in the molecule or than the same sites in other SKs that activate non-human Pgs.

Another important aspect of species specificity is to understand how a microorganism, adapted to a given species, may breach the species barrier and establish an infection in another species. This question is of particular interest regarding the adaptation of SIV from non-human primates to HIV in humans. This topic is also particularly relevant to the recent outbreak of acute respiratory syndrome due to the SARS coronavirus, and the flu pandemics caused by viruses of swine and avian origin [90].

References

1 von Bernuth, H., Picard, C., Jin, Z., Pankla, R., Xiao, H., Ku, C.L., *et al.* (2008) Pyogenic bacterial infections in humans with MyD88 deficiency. *Science*, **321**, 691–696.

2 Mengaud, J., Ohayon, H., Gounon, P., Mege, R.M., and Cossart, P. (1996) E-cadherin is the receptor for internalin, a surface protein required for entry of L. monocytogenes into epithelial cells. *Cell*, **84**, 923–932.

3 Zhang, J.R., Mostov, K.E., Lamm, M.E., Nanno, M., Shimida, S., Ohwaki, M., and Tuomanen, E. (2000) The polymeric immunoglobulin receptor translocates pneumococci across human nasopharyngeal epithelial cells. *Cell*, **102**, 827–837.

4 Ploss, A., Evans, M.J., Gaysinskaya, V.A., Panis, M., You, H., de Jong, Y.P., and Rice, C.M. (2009) Human occludin is a hepatitis C virus entry factor required for infection of mouse cells. *Nature*, **457**, 882–886.

5 Evans, M.J., von Hahn, T., Tscherne, D.M., Syder, A.J., Panis, M., Wolk, B., *et al.* (2007) Claudin-1 is a hepatitis C virus co-receptor required for a late step in entry. *Nature*, **446**, 801–805.

6 Pileri, P., Uematsu, Y., Campagnoli, S., Galli, G., Falugi, F., Petracca, R., *et al.* (1998) Binding of hepatitis C virus to CD81. *Science*, **282**, 938–941.

7 Shelburne, S.A., 3rd, Sumby, P., Sitkiewicz, I., Granville, C., DeLeo, F.R., and Musser, J.M. (2005) Central role of a bacterial two-component gene regulatory system of previously unknown function in pathogen persistence in human saliva. *Proc. Natl Acad. Sci. USA*, **102**, 16037–16042.

8 Shelburne, S.A., 3rd, Granville, C., Tokuyama, M., Sitkiewicz, I., Patel, P., and Musser, J.M. (2005) Growth characteristics of and virulence factor production by group A Streptococcus during cultivation in human saliva. *Infect. Immun.*, **73**, 4723–4731.

9 Coron, E., Flamant, M., Aubert, P., Wedel, T., Pedron, T., Letessier, E., *et al.* (2009) Characterisation of early mucosal and neuronal lesions following Shigella flexneri infection in human colon. *PLoS ONE*, **4**, e4713.

10 Lecuit, M., Nelson, D.M., Smith, S.D., Khun, H., Huerre, M., Vacher-Lavenu, M.C., *et al.* (2004) Targeting and crossing of the human maternofetal barrier by Listeria monocytogenes: role of internalin interaction with trophoblast E-cadherin. *Proc. Natl Acad. Sci. USA*, **101**, 6152–6157.

11 Bukh, J. (2004) A critical role for the chimpanzee model in the study of hepatitis C. *Hepatology*, **39**, 1469–1475.

12 Virtaneva, K., Graham, M.R., Porcella, S.F., Hoe, N.P., Su, H., Graviss, E.A., *et al.* (2003) Group A Streptococcus gene expression in humans and cynomolgus macaques with acute pharyngitis. *Infect. Immun.*, **71**, 2199–2207.

13 Sasaki, E., Suemizu, H., Shimada, A., Hanazawa, K., Oiwa, R., Kamioka, M., *et al.* (2009) Generation of transgenic non-human primates with germline transmission. *Nature*, **459**, 523–527.

14 Disson, O., Grayo, S., Huillet, E., Nikitas, G., Langa-Vives, F., Dussurget, O., *et al.* (2008) Conjugated action of two species-specific invasion proteins for fetoplacental listeriosis. *Nature*, **455**, 1114–1118.

15 Watanabe, T., Tada, M., Nagai, H., Sasaki, S., and Nakao, M. (1998) Helicobacter pylori infection induces gastric cancer in Mongolian gerbils. *Gastroenterology*, **115**, 642–648.

16 Ogura, K., Maeda, S., Nakao, M., Watanabe, T., Tada, M., Kyutoku, T., *et al.* (2000) Virulence factors of Helicobacter pylori responsible for gastric diseases in Mongolian gerbil. *J. Exp. Med.*, **192**, 1601–1610.

17 Davis, J.M., and Ramakrishnan, L. (2009) The role of the granuloma in expansion and dissemination of early tuberculous infection. *Cell*, **136**, 37–49.

18 Monack, D.M., Mueller, A., and Falkow, S. (2004) Persistent bacterial infections: the interface of the pathogen and the host immune system. *Nat. Rev. Microbiol.*, **2**, 747–765.

19 Grassl, G.A., and Finlay, B.B. (2008) Pathogenesis of enteric Salmonella infections. *Curr. Opin. Gastroenterol.*, **24**, 22–26.

20 Marchetti, M., Arico, B., Burroni, D., Figura, N., Rappuoli, R., and Ghiara, P. (1995) Development of a mouse model of *Helicobacter pylori* infection that mimics human disease. *Science*, **267**, 1655–1658.

21 Wollert, T., Pasche, B., Rochon, M., Deppenmeier, S., van den Heuvel, J., Gruber, A.D., *et al.* (2007) Extending the host range of *Listeria monocytogenes* by rational protein design. *Cell*, **129**, 891–902.

22 Davis, P.H., and Stanley, S.L., Jr. (2003) Breaking the species barrier: use of SCID mouse-human chimeras for the study of human infectious diseases. *Cell. Microbiol.*, **5**, 849–860.

23 Bosma, G.C., Custer, R.P., and Bosma, M.J. (1983) A severe combined immunodeficiency mutation in the mouse. *Nature*, **301**, 527–530.

24 Mosier, D.E., Gulizia, R.J., Baird, S.M., and Wilson, D.B. (1988) Transfer of a functional human immune system to mice with severe combined immunodeficiency. *Nature*, **335**, 256–259.

25 Mosier, D.E., Gulizia, R.J., Baird, S.M., Wilson, D.B., Spector, D.H., and Spector, S.A. (1991) Human immunodeficiency virus infection of human-PBL-SCID mice. *Science*, **251**, 791–794.

26 Mercer, D.F., Schiller, D.E., Elliott, J.F., Douglas, D.N., Hao, C., Rinfret, A., *et al.* (2001) Hepatitis C virus replication in mice with chimeric human livers. *Nat. Med.*, **7**, 927–933.

27 Zhang, Z., Jin, L., Champion, G., Seydel, K.B., and Stanley, S.L., Jr. (2001) Shigella infection in a SCID mouse-human intestinal xenograft model: role for neutrophils in containing bacterial dissemination in human intestine. *Infect. Immun.*, **69**, 3240–3247.

28 Sperandio, B., Regnault, B., Guo, J., Zhang, Z., Stanley, S.L., Jr., Sansonetti, P.J., and Pedron, T. (2008) Virulent *Shigella flexneri* subverts the host innate immune response through manipulation of antimicrobial peptide gene expression. *J. Exp. Med.*, **205**, 1121–1132.

29 Lozniewski, A., Muhale, F., Hatier, R., Marais, A., Conroy, M.C., Edert, D., *et al.* (1999) Human embryonic gastric xenografts in nude mice: a new model of *Helicobacter pylori* infection. *Infect. Immun.*, **67**, 1798–1805.

30 Scaramuzzino, D.A., McNiff, J.M., and Bessen, D.E. (2000) Humanized *in vivo* model for streptococcal impetigo. *Infect. Immun.*, **68**, 2880–2887.

31 Ren, R.B., Costantini, F., Gorgacz, E.J., Lee, J.J., and Racaniello, V.R. (1990) Transgenic mice expressing a human poliovirus receptor: a new model for poliomyelitis. *Cell*, **63**, 353–362.

32 Zhang, S., and Racaniello, V.R. (1997) Expression of the poliovirus receptor in intestinal epithelial cells is not sufficient to permit poliovirus replication in the mouse gut. *J. Virol.*, **71**, 4915–4920.

33 Ohka, S., Igarashi, H., Nagata, N., Sakai, M., Koike, S., Nochi, T., *et al.* (2007) Establishment of a poliovirus oral infection system in human poliovirus receptor-expressing transgenic mice that are deficient in alpha/beta interferon receptor. *J. Virol.*, **81**, 7902–7912.

34 Lecuit, M., Vandormael-Pournin, S., Lefort, J., Huerre, M., Gounon, P., Dupuy, C., *et al.* (2001) A transgenic model for listeriosis: role of internalin in crossing the intestinal barrier. *Science*, **292**, 1722–1725.

35 Johansson, L., Rytkonen, A., Bergman, P., Albiger, B., Kallstrom, H., Hokfelt, T., *et al.* (2003) CD46 in meningococcal disease. *Science*, **301**, 373–375.

36 Sun, H., Ringdahl, U., Homeister, J.W., Fay, W.P., Engleberg, N.C., Yang, A.Y., *et al.* (2004) Plasminogen is a critical host pathogenicity factor for group A streptococcal infection. *Science*, **305**, 1283–1286.

37 Hamon, M., Bierne, H., and Cossart, P. (2006) *Listeria monocytogenes*: a multifaceted model. *Nat. Rev. Microbiol.*, **4**, 423–434.

38 Pirie, J. (1927) A new disease of veld rodents, 'Tiger river disease'. *Pub. S. Afr. Inst. Med. Res.*, **62**, 163–186.

39 Vazquez-Boland, J.A., Kuhn, M., Berche, P., Chakraborty, T., Dominguez-Bernal, G., Goebel, W., *et al.* (2001) Listeria pathogenesis and molecular virulence determinants. *Clin. Microbiol. Rev.*, **14**, 584–640.

40 Pamer, E.G. (2004) Immune responses to *Listeria monocytogenes*. *Nat. Rev. Immunol.*, **4**, 812–823.

41 Yano, T., Mita, S., Ohmori, H., Oshima, Y., Fujimoto, Y., Ueda, R., *et al.* (2008) Autophagic control of Listeria through intracellular innate immune recognition in drosophila. *Nat. Immunol.*, **9**, 908–916.

41a Levraud, J.P., Disson, O., Kissa, K., Bonne, I., Cossart, P., Herbomel, P., and Lecuit, M. (2009) Real-time observation of listeria monocytogenes-phagocyte interactions in living zebrafish larvae. *Infect. Immun*, **9**, 3651–3660.

42 Lecuit, M., Dramsi, S., Gottardi, C., Fedor-Chaiken, M., Gumbiner, B., and Cossart, P. (1999) A single amino acid in E-cadherin responsible for host specificity towards the human pathogen *Listeria monocytogenes*. *EMBO J.*, **18**, 3956–3963.

43 Schubert, W.D., Urbanke, C., Ziehm, T., Beier, V., Machner, M.P., Domann, E., *et al.* (2002) Structure of internalin, a major invasion protein of *Listeria monocytogenes*, in complex with its human receptor E-cadherin. *Cell*, **111**, 825–836.

44 Bonazzi, M., Lecuit, M., and Cossart, P. (2009) Listeria monocytogenes internalin and E-cadherin: from structure to pathogenesis. *Cell. Microbiol.*, **11**, 693–702.

45 Shen, Y., Naujokas, M., Park, M., and Ireton, K. (2000) InIB-dependent internalization of Listeria is mediated by the Met receptor tyrosine kinase. *Cell*, **103**, 501–510.

46 Khelef, N., Lecuit, M., Bierne, H., and Cossart, P. (2006) Species specificity of the *Listeria monocytogenes* InlB protein. *Cell. Microbiol.*, **8**, 457–470.

47 Niemann, H.H., Jager, V., Butler, P.J., van den Heuvel, J., Schmidt, S., Ferraris, D., *et al.* (2007) Structure of the human receptor tyrosine kinase Met in complex with the Listeria invasion protein InlB. *Cell*, **130**, 235–246.

48 Nunes, M.C., and Scherf, A. (2007) *Plasmodium falciparum* during pregnancy: a puzzling parasite tissue adhesion tropism. *Parasitology*, **134**, 1863–1869.

49 Gerardin, P., Barau, G., Michault, A., Bintner, M., Randrianaivo, H., Choker, G., *et al.* (2008) Multidisciplinary prospective study of mother-to-child chikungunya virus infections on the island of La Reunion. *PLoS Med.*, **5**, e60.

50 Lecuit, M. (2005) Understanding how *Listeria monocytogenes* targets and crosses host barriers. *Clin. Microbiol. Infect.*, **11**, 430–436.

51 Barragan, A., and Sibley, L.D. (2003) Migration of *Toxoplasma gondii* across biological barriers. *Trends Microbiol.*, **11**, 426–430.

52 Pereira, L., Maidji, E., McDonagh, S., and Tabata, T. (2005) Insights into viral transmission at the uterine-placental interface. *Trends Microbiol.*, **13**, 164–174.

53 Banatvala, J.E., and Brown, D.W. (2004) Rubella. *Lancet*, **363**, 1127–1137.

54 Bakardjiev, A.I., Stacy, B.A., Fisher, S.J., and Portnoy, D.A. (2004) Listeriosis in the pregnant guinea pig: a model of vertical transmission. *Infect. Immun.*, **72**, 489–497.

55 Le Monnier, A., Autret, N., Join-Lambert, O.F., Jaubert, F., Charbit, A., Berche, P., and Kayal, S. (2007) ActA is required for crossing of the fetoplacental barrier by *Listeria monocytogenes*. *Infect. Immun.*, **75**, 950–957.

56 Cunningham, M.W. (2000) Pathogenesis of group A streptococcal infections. *Clin. Microbiol. Rev.*, **13**, 470–511.

57 McCoy, H.E., Broder, C.C., and Lottenberg, R. (1991) Streptokinases produced by pathogenic group C streptococci demonstrate species-specific plasminogen activation. *J. Infect. Dis.*, **164**, 515–521.

58 Olsen, R.J., Shelburne, S.A., and Musser, J.M. (2009) Molecular mechanisms underlying group A streptococcal pathogenesis. *Cell Microbiol.*, **11**, 1–12.

59 Ringdahl, U., Svensson, M., Wistedt, A.C., Renne, T., Kellner, R., Muller-Esterl, W., and Sjobring, U. (1998) Molecular co-operation between protein PAM and streptokinase for plasmin acquisition by *Streptococcus pyogenes*. *J. Biol. Chem.*, **273**, 6424–6430.

60 Walker, M.J., McArthur, J.D., McKay, F., and Ranson, M. (2005) Is plasminogen deployed as a *Streptococcus pyogenes* virulence factor? *Trends Microbiol.*, **13**, 308–313.

61 Fu, Q., Figuera-Losada, M., Ploplis, V.A., Cnudde, S., Geiger, J.H., Prorok, M., and Castellino, F.J. (2008) The lack of binding of VEK-30, an internal peptide from the group A streptococcal M-like protein, PAM, to murine plasminogen is due to two amino acid replacements in the plasminogen kringle-2 domain. *J. Biol. Chem.*, **283**, 1580–1587.

62 Svensson, M.D., Scaramuzzino, D.A., Sjobring, U., Olsen, A., Frank, C., and Bessen, D.E. (2000) Role for a secreted cysteine proteinase in the establishment of host tissue tropism by group A streptococci. *Mol. Microbiol.*, **38**, 242–253.

63 Svensson, M.D., Sjobring, U., Luo, F., and Bessen, D.E. (2002) Roles of the plasminogen activator streptokinase and the plasminogen-associated M protein in an experimental model for streptococcal impetigo. *Microbiology*, **148**, 3933–3945.

64 Khil, J., Im, M., Heath, A., Ringdahl, U., Mundada, L., Cary Engleberg, N., and Fay, W.P. (2003) Plasminogen enhances virulence of group A streptococci by streptokinase-dependent and streptokinase-independent mechanisms. *J. Infect. Dis.*, **188**, 497–505.

65 Sun, H., Wang, X., Degen, J.L., and Ginsburg, D. (2009) Reduced thrombin generation increases host susceptibility to group A streptococcal infection. *Blood*, **113**, 1358–1364.

66 Ashbaugh, C.D., Moser, T.J., Shearer, M.H., White, G.L., Kennedy, R.C., and Wessels, M.R. (2000) Bacterial determinants of persistent throat colonization and the associated immune response in a primate model of human group A streptococcal pharyngeal infection. *Cell. Microbiol.*, **2**, 283–292.

67 Virtaneva, K., Porcella, S.F., Graham, M.R., Ireland, R.M., Johnson, C.A., Ricklefs, S.M., *et al.* (2005) Longitudinal analysis of the group A Streptococcus transcriptome in experimental pharyngitis in cynomolgus macaques. *Proc. Natl Acad. Sci. USA*, **102**, 9014–9019.

68 Elm, C., Braathen, R., Bergmann, S., Frank, R., Vaerman, J.P., Kaetzel, C.S., *et al.* (2004) Ectodomains 3 and 4 of human polymeric Immunoglobulin receptor (hpIgR) mediate invasion of *Streptococcus pneumoniae* into the epithelium. *J. Biol. Chem.*, **279**, 6296–6304.

69 Sun, K., Johansen, F.E., Eckmann, L., and Metzger, D.W. (2004) An important role for polymeric Ig receptor-mediated transport of IgA in protection against *Streptococcus pneumoniae* nasopharyngeal carriage. *J. Immunol.*, **173**, 4576–4581.

70 Lu, L., Ma, Z., Jokiranta, T.S., Whitney, A.R., DeLeo, F.R., and Zhang, J.R. (2008) Species-specific interaction of *Streptococcus pneumoniae* with human complement factor H. *J. Immunol.*, **181**, 7138–7146.

71 Gill, D.B., and Atkinson, J.P. (2004) CD46 in Neisseria pathogenesis. *Trends Mol. Med.*, **10**, 459–465.

72 Dorig, R.E., Marcil, A., Chopra, A., and Richardson, C.D. (1993) The human CD46 molecule is a receptor for measles virus (Edmonston strain). *Cell*, **75**, 295–305.

73 Santoro, F., Kennedy, P.E., Locatelli, G., Malnati, M.S., Berger, E.A., and Lusso, P. (1999) CD46 is a cellular receptor for human herpesvirus 6. *Cell*, **99**, 817–827.

74 Gaggar, A., Shayakhmetov, D.M., and Lieber, A. (2003) CD46 is a cellular receptor for group B adenoviruses. *Nat. Med.*, **9**, 1408–1412.

75 Okada, N., Liszewski, M.K., Atkinson, J.P., and Caparon, M. (1995) Membrane cofactor protein (CD46) is a keratinocyte receptor for the M protein of the group A streptococcus. *Proc. Natl Acad. Sci. USA*, **92**, 2489–2493.

76 Kallstrom, H., Liszewski, M.K., Atkinson, J.P., and Jonsson, A.B. (1997) Membrane cofactor protein (MCP or CD46) is a cellular pilus receptor for pathogenic Neisseria. *Mol. Microbiol.*, **25**, 639–647.

77 Johansson, L., Rytkonen, A., Wan, H., Bergman, P., Plant, L., Agerberth, B., *et al.* (2005) Human-like immune responses in CD46 transgenic mice. *J. Immunol.*, **175**, 433–440.

78 Kirchner, M., Heuer, D., and Meyer, T.F. (2005) CD46-independent binding of neisserial type IV pili and the major pilus adhesin, PilC, to human epithelial cells. *Infect. Immun.*, **73**, 3072–3082.

79 Tobiason, D.M., and Seifert, H.S. (2001) Inverse relationship between pilus-mediated gonococcal adherence and surface expression of the pilus receptor, CD46. *Microbiology*, **147**, 2333–2340.

80 Mairey, E., Genovesio, A., Donnadieu, E., Bernard, C., Jaubert, F., Pinard, E., *et al.* (2006) Cerebral microcirculation shear stress levels determine *Neisseria meningitidis* attachment sites along the blood–brain barrier. *J. Exp. Med.*, **203**, 1939–1950.

81 Coureuil, M., Mikaty, G., Miller, F., Lécuyer, H., Bernard, C., Bourdoulous, S., *et al.* (2009) Meningococcal type IV pili recruit the polarity complex to cross the brain endothelium. *Science*, **325**, 83–87.

82 Orihuela, C.J., Mahdavi, J., Thornton, J., Mann, B., Wooldridge, K.G., Abouseada, N., *et al.* (2009) Laminin receptor initiates bacterial contact with the blood brain barrier in experimental meningitis models. *J. Clin. Invest.*, **119**, 1638–1646.

83 Stojiljkovic, I., Larson, J., Hwa, V., Anic, S., and So, M. (1996) HmbR outer membrane receptors of pathogenic Neisseria spp.: iron-regulated, hemoglobin-binding proteins with a high level of primary structure conservation. *J. Bacteriol.*, **178**, 4670–4678.

84 Zarantonelli, M.L., Szatanik, M., Giorgini, D., Hong, E., Huerre, M., Guillou, F., *et al.* (2007) Transgenic mice expressing human transferrin as a model for meningococcal infection. *Infect. Immun.*, **75**, 5609–5614.

85 Kwiatkowski, D.P. (2005) How malaria has affected the human genome and what human genetics can teach us about malaria. *Am. J. Hum. Genet.*, **77**, 171–192.

86 Fischer, A. (2007) Human primary immunodeficiency diseases. *Immunity*, **27**, 835–845.

87 Arbibe, L., Kim, D.W., Batsche, E., Pedron, T., Mateescu, B., Muchardt, C., *et al.* (2007) An injected bacterial effector targets chromatin access for transcription factor NF-kappaB to alter transcription of host genes involved in immune responses. *Nat. Immunol.*, **8**, 47–56.

88 Hamon, M.A., Batsche, E., Regnault, B., Tham, T.N., Seveau, S., Muchardt, C.,

and Cossart, P. (2007) Histone modifications induced by a family of bacterial toxins. *Proc. Natl Acad. Sci. USA*, **104**, 13467–13472.

89 Gladysheva, I.P., Turner, R.B., Sazonova, I.Y., Liu, L., and Reed, G.L. (2003) Coevolutionary patterns in plasminogen activation. *Proc. Natl Acad. Sci. USA*, **100**, 9168–9172.

90 Neumann, G., Noda, T., and Kawaoka, Y. (2009) Emergence and pandemic potential of swine-origin H1N1 influenza virus. *Nature*, **459**, 931–939.

Index

a

a proliferation-inducing ligand (APRIL) 221ff.
ABC transport system 8
Abelson-interacting protein-1 (AbI-1) 178
accessory region (AR) 107
N-acetylmuramoyl-L-alanine amidase (LytA) 113
activation-induced cytidine deaminase (AID) 36
acute lower respiratory tract infection (ARI) 103
acute otitis media (AOM) 107
acyloxyacyl hydrolase (AOAH) 233
adaptive capability
– bacteria 3
adhesin 300
adhesion
– disorder 26
AIM2 237
airway 254
angiogenin Ang-4 222
animal model 282
– genetic engineering 286
– humanized 284ff.
AnkH 126
AnkJ 126
antigen
– pro-inflammatory 233
antigen sampling
– gut 226
antimicrobial peptide 46, 254
antimicrobials 222f.
argonaut (Ago) family 175
Aspergillus nidulans 268
asymptomatic bacteriuria (ABU) 9
azurocidin 23

b

B cell 229
B cell-activating factor (BAFF) 229
Bacillus anthracis 255
bacteria
– adaptive capability 3
– blood-borne 86
– extravasation 88
– population dynamics in the murine host 156
bacterial gene
– virulence 146
bacterial genome optimization
– *E. coli* as model 7
bacterial infection 139
– mouse model 139
– species specificity 279ff.
bacterial invasion
– modelization 281
bacterial pathogen 3, 103
– community acquired pneumonia (CAP) 103ff.
– passage of extracellular bacterial pathogen across the blood–CSF barrier 75
bacterial resistance mechanism 46
bacterial translocation
– central nervous system vasculature 70
bacterial virulence factor 112
bacterial virulence gene 147
bactericidal/permeability-increasing protein (BPI) 23
bacteriocin 111
Bacteroides fragilis 204
Bacteroides thetaiotaomicron 204
Bacteroidetes 200f., 233
bacterium
– modifying 284
beta-*N*-acetylglucosamidase 113

Bacterial Virulence: Basic Principles, Models and Global Approaches. Edited by Philippe Sansonetti
© 2010 WILEY-VCH Verlag GmbH & Co. KGaA, Weinheim
ISBN: 978-3-527-32326-5

bioluminescence (BLI) 255ff.
blood
– *ex vivo* infection 281
blood–brain barrier (BBB) 69ff., 287, 301ff.
– anatomy 71
blood–CNS interface 72
– structural and functional heterogeneity 70
blood–CSF barrier 74
– passage of extracellular bacterial pathogen 75f.
blpMN gene 111
body fluid
– *ex vivo* infection 281
brain vessel
– extravasation of bacteria 88
bundle-forming pilus (Bfp1) 258

c

C-reactive protein (CRP) 110
C-terminal Src kinase (Csk) 35
C3b receptor (CR3) 54
C3bi complement recognition 21
C5a 50ff.
C5a receptor (C5aR) 54
Ca^{2+} concentration 24
CagA 34
cag pathogenicity island (*cagPAI*) 34
calprotectin 57
CAMP β-defensin-3 47
Campylobacter jejuni 256
canceled-out index (COI) 148
catalase (KatA) 56
β-catenin 35
cathelicidin 222f.
cationic antimicrobial peptide (CAMP) 47
CbpA 300
CbpA–hpIgR 280
CC chemokine ligand (CCL)
– CCL20 221
– *CCL20* 285
$CD4^+$ T cell 231
$CD4^+$ T helper (Th) cell 229
CD4T cell
– Th1-type 32
CD11b 227
CD8 227
CD18 26
CD18-expressing phagocyte 152
CD18/CD11a 19ff.
CD18/CD11b 19ff.
CD18/CD11c 26
CD40 ligand (CD40L) 229
CD44 89f.
CD46 280, 302
CD81 280
CD103 227
CD155 286
Cdc2-like kinase Clk1 127
Cdc42 Rho GTPase 90
CDR3 53
Ceg9 123
Ceg19 123
cell membrane glycolipid anchor LTA 84
central nervous system (CNS) 69
– vasculature 70
cerebrospinal meningitis 84, 301
Chédiak-Higashi syndrome 27
chemokine 48f., 221
chemotaxis
– disorder 26
CHIPS (chemotaxis inhibitory protein of staphylococci) 54
Chlamydia 165ff.
– genetic determinants of infection and disease 172
– host cell factors during infection 174
– host interaction 166ff.
– host susceptibility to infection 179
– intracellular pathogen–host relationship 167
– *muridarum* 167
– *psittaci* 167
– RNAi 178
– spp 103
– therapeutic discovery 179
Chlamydia trachomatis 166
– attachment and invasion 170
– cycle of development 168
– expansion and nutrient acquisition 170
– host cell exit 171
– inclusion formation 170
– persistence 171
chlamydial factor 172
chlamydial protease-like activity factor (CPAF) 171
cholera toxin 5
choline-blinding protein (CBP) 113
– CbpA 110ff.
– CbpD 114
– CbpE 113f.
choroids plexus (CPs) 74ff.
chromatin 25
chronic atrophic gastritis (ChAG) 10
chronic granulomatous disease (CGD) 27
CHS1 27
circling disease 288
Citrobacter 260

class switch reaction (CSR) 229
claudin-1 280
collagenase 24
colonization factor antigen (Cfa) 258
ComD1 109
ComD2 109
community-acquired (CA)-MRSA 45, 57
community acquired pneumonia (CAP) 103ff.
– bacterial pathogen 103
competence (*com*)
– *comAB* 109
– *comCDE* 109
– *comW* 109
competence and sporulation factor (CSF) 235
competence-stimulating pheromone (CSP) 109
competitive index (CI) test 146
complement factor H (FH) 301
CRAMP 47
Crohn's disease (CD) 224
crypt lumen 222
cryptdin 222
CX_3CR1 227
CXC chemokine 52
CXCL1 48
CXCL2 48
CXCL6 granulocytes chemotactic protein 2 (GCP-2) 52
CXCL8 49, 221, 239
CXCR1 263
CXCR2/IL-8R 52
cystic fibrosis (CF) 9
cytokine 231
– pro-inflammatory 49, 221
cytophaga-flavobacterium-bacteroides (CFB) bacteria 232
cytosolic flagellin
– Naip-5-dependent immune response 130
cytotoxic necrotizing factor-1 (CNF-1) 81, 263

d

α-defensin 23, 222ff.
– human α-defensin (HD5) 222
β-defensin 222f.
– 3 47
degranulation 17ff.
– disorder 27
dendritic cell (DC) 199, 221ff.
DICER 175
Dictyostelium
– *discoideum* 130
– transcriptional host cell response 130
differential killing hypothesis 209
diphtheria toxin 5
DNA 25
– aberrant methylation by *H. pylori* 37
Dot/Icm type-IV secretion system 121ff.

e

E-cadherin (Ecad) 280ff.
E-cadherin–internalin (Ecad-InlA) 280
early endosomal antigen 1 (EEA1) 126
ecto-nucleoside triphosphate diphosphohydrolase (ecto-NTPDase) 125
eitA-D genes 8
elementary body (EB) 167
enabling avoidance of killing 147
endothelial docking structure 90
Enteroaceriaceae family 233
Enterobacter 263
Enterobacteriaceae 79, 256
Enterococcus faecalis 263
enteropathogen 209
eosinophil 225
epidermal growth factor (EGF) receptor 90
ErbB2 90
ERM (ezrin, radixin, moesin) protein 89
euchromatin 25
eukaryotic-like protein 127
Escherichia coli 7, 69ff.
– avian pathogenic (APEC) 8
– bacterial genome optimization 7
– enterohemorrhagic (EHEC) 7, 218, 256
– enteroinvasive (EIEC) 256
– enteropathogenic (EPEC) 7, 256
– enterotoxigenic (ETEC) 7, 256
– extraintestinal pathogenic (ExPEC) 7, 79
– IPEC 7
– K1 69ff.
– meningitis 79
– strains causing neonatal meningitis (ECNM) 79f.
– uropathogenic (UPEC) 263
ex vivo model 299
exocytosis
– secretory vesicles 22
extracellular adherence protein (Eap) 53
extravasation
– bacteria 88
ezrin 89f.

f

facilitating replication 147
factor-H binding protein (fHBP) 85f.
Fc receptor (FcRn) 227

Fcα receptor 228
Fcγ receptor 21
fibered confocal fluorescence microscopy (FCFM) 256
Firmicutes 200f.
flexible gene pool
– composition 4
fluorescence recovery after photobleaching (FRAP) 252
fluorescence resonance energy transfer (FRET) 252ff.
[18F]fluorodeoxyglucose (FDG) 255
– FDG-PET 255
fMLP 235
focal adhesion kinase (FAK) 35
follicle-associated epithelium (FAE) 226
food hypothesis 209
forkhead box P3 (FoxP3) 231
formylated peptide receptor (FPR) 54
fusion 24

g

gastric cancer 37
gastric carcinogenesis 34
– *Heliobacter pylori*-induced 31
gastric epithelial cell 31f.
gastritis 31
gastroenteritis 289
gastrointestinal microbiota 196ff.
– acquisition 201
– diversity 199
gastrointestinal (GI) tract 256
– infection 256
Gbs1474 84
Gbs1477 84
Gbs1478 84
GDP association inhibitor (GDI) 122
gelatinase 24
gene mutation 35
genome 3
genome dynamics
– mechanism 6
genome plasticity
– infection 9
genomic island (GEI) 4ff.
gliovascular unit 73
glucose-6-phosphate dehydrogenase (G6PD) deficiency 27f.
glutathione peroxidase (GpoA) 55
glycosaminoglycan (GAG) 170
Goblet cell 218ff.
$gp22^{phox}$ 27
$gp47^{phox}$ 27
$gp67^{phox}$ 27
$gp91^{phox}$, *see* Nox2
$gp91phox^{-/-}$ mouse 153
granular protein 25
granules 23
green fluorescent protein (GFP) 252
group A *Streptococcus* (GAS) 45ff., 281, 296ff.
– avoidance of clearance by PMN 50
– GAS cysteine protease (SpeB) 50ff.
– restriction of cutaneous infection 46
group B *Streptococcus* (GBS) 69, 82
– meningitis 82
growth related oncogene alpha (GROα) 52
guanine nucleotide exchange factor (GEF) 126
gut
– antigen sampling 226
gut barrier
– anatomy 215f.
– physiological and immunological property 216
gut epithelium architecture 205
gut homeostasis 232
gut microbiota 195ff.
– immune tolerance 207
– infection and transmission of pathogen 207
– symbiosis with host 203
gut-associated lymphoid tissue (GALT) 203, 216

h

Haemophilus influenzae 103ff.
– type b 69
HBD-3 47
HD5 222
Helicobacter mustelae 283
Helicobacter pylori 31ff., 283
– aberrant DNA methylation 37
– *cag* pathogenicity island (*cagPAI*) 34
– gene mutation 35
– infection 31ff.
– relationship between direct and indirect actions 37
β-hemolysin/cytolysin (β-h/c) 83
heparin-binding protein (HBP) 50
heterochromatin 25
HIP/PAP 222
histone 25
homeostasis 195ff., 238
– intestinal 215ff., 238
horizontal gene transfer (HGT) 127
host
– symbiosis with gut microbiota 203

– pathogen factor 148
host cell factor
– *Chlamydia* infection 174
host cell modulation 125
host cell response 130
host defense
– invading microbe 117
host factor 173
host interaction
– *Chlamydia* 166
host tissue
– cell interaction 153
host–pathogen interaction
– imaging 251ff.
host–pathogen relationship 45ff.
human leukocyte antigen (HLA) 173
human neutrophil peptide 23
human peptide transporter 1 (hPepT1) 235
human poliovirus receptor (hPVR) 286
hydrogen peroxide 117
hyperimmunoglobulin E (hyper-IgE) syndrome 27

i

IgA
– secretory IgA (SIgA) 228
IgA response
– protective 228
IgA1-protease 115
Ihk/Irr 55
IκB α 239
IκB kinase (IKK) complex 239f.
– IKKα 240
– IKKβ 240
– IKKγ 240
imaging
– clinical implication 268
– dynamic technology 251ff.
– host–pathogen interaction 251
– infection 253
immune cell 17
immune tolerance
– gut microbiota 207
immunoglobulin
– secreted (IgA) 254
immunoglobulin receptor
– polymeric (pIgR) 228
in vivo expression technology (IVET) 143
in vivo model 300
in vivo modeling 282ff.
inducible-nitric-oxide-synthase (iNOS) 227
– $iNOS^{-/-}$ mouse 153
infection
– GI tract 256, 258ff.
– *Helicobacter pylori* 31ff.
– imaging 253
– neutrophil recruitment 18
– systemic 296
inflammation 209
– intestinal 237
– route to cancer 31
inflammatory bowel disease (IBD) 207f., 216ff., 242
influenza virus
– pneumococci 111
ingestion
– disorder 27
inhibitor of apoptosis (IAP) 178
InlB 280ff.
– InlB–Met interaction 289ff.
innate immune cell 224
innate immunity 48
integrative and conjugative element (ICE) 4ff.
β_2-integrin 19ff., 53
integron 4
intercellular adhesion molecule (ICAM-1) 53, 89f.
interferon γ (IFN-γ) 32, 231
interferon regulatory factor (IRF) family 236
interleukin 1 receptor (IL-1R) 49
interleukin
– IL-β 32, 78
– IL-4 231
– IL-5 231
– IL-6 33, 49, 78, 221
– IL-8 49ff.
– IL-10 231f.
– IL-11 33
– IL-12 49
– IL-13 231
– IL-17A 231
– IL-17F 231
– IL-21 231
– IL-22 231
internalin
– InlA 280ff.
– InlA–Ecad interaction 280ff.
intestinal alkaline phosphatase (IAP) 234
intestinal bacterial community 202
intestinal barrier (IB) 287
intestinal crypt 222
intestinal epithelial cell (IEC) 221ff.
intestinal fatty-acid-binding protein (iFABP) gene 291
intestinal homeostasis 215
intestinal infection 287

intestinal lumen
– oxygen availability 218
IPAF 237
IPEC 7
IRAK-M 239
iro determinant 8
IS-element 4
isolated (solitary) lymphoid follicle (ILF) 206, 216

j
jumping gene 4

k
keratinocyte-derived chemokine (KC) 48ff.
Klebsiella 263
– *pneumoniae* 254
knock-in mouse 287

l
lactoferrin 23
lamina propria (LP) 206, 216, 224
laminin receptor (LR) 304
laser scanning confocal microscopy (LSCM) 252
Legionella 104, 120f.
– genome 128
– host response 129
– pathogenesis 121
– spp 103
Legionella pneumophila 103ff., 119ff.
– MyD88 response 129
– phagosomal–lysosomal fusion 125
– virulence strategy 124
Legionella-containing vacuole (LCV) 122ff.
legionellosis 119
Legionnaires' disease (LD) 119f.
– epidemiology 119
LepA 127
LepB 127
leukocyte 47
leukocyte adhesion deficiency (LAD) 26
leukolysin 24
lipid droplet (LD) 170
lipopolysaccharide (LPS) 233, 263
Listeria
– *innocua* 289
– model of intestinal and materno-fetal infection 287
– *monocytogenes* 238, 267, 281ff.
listeriolysin (LLO) 288
localized juvenile periodontitis (LJP) 27
LPxTG motif 112ff.
LPXTG protein 110
lung infection 254
lymph node (LN) 216
lymphocyte function antigen (LFA-1) 19ff., 26, 53
lymphoid tissue
– maturation 206
lysozyme 254
– LytC 113f.
LytA 111ff.
LytB 113f.

m
M cell 226
M-protein 54
macroevolution 10
macrophage 46
– Mφ 58f., 224
macrophage antigen-1 (Mac-1) 19ff.
magnetic resonance imaging (MRI) 255
major histocompatibility (MHC) complex II 262
major outer membrane protein (MOMP) 167ff.
Marco 118
materno-fetal barrier (MFB) 287
materno-fetal infection 287ff.
matrix-degrading metalloprotease (MMP) 24
– MMP-9 116
membrane cofactor protein (MCP) 88, 302
meningeal invasion 69ff.
– septicemic extracellular pathogen 69ff.
meninges 71
meningitis 78f., 267, 302
– cerebrospinal 84, 301
– *E. coli* 79
– early onset disease (EOD) 82
– group B *Streptococcus* (GBS) 82f.
– late onset disease (LOD) 82
– neonatal 79ff.
meningococcal infection 304
Met 280ff.
methicillin-resistant SA (MRSA) 45
microbe
– sensing the presence 235ff.
microbe-associated molecular pattern (MAMP) 228ff.
– MAMP-TLR recognition 236
microbial diversity 196
microbial surface component recognizing adhesive matrix molecules (MSCRAMM) 113
microbiota 195ff.
– metabolic function 204

– sensing, signaling and responding 232
microevolution 10
MIP-2 48ff.
mitogen-activated protein kinase (MAPK) 235
– pathway 236
mobilization 24
moesin 89f.
Moraxella catarrhalis 103
mouse gene knockout 149ff.
mucin
– MUC2 220
mucosal epithelium 220
– immune function 220
mucus layer 218
multi-locus sequence typing (MLST) 80, 107
multi-photon-based intravital microscopy 266
muramyl dipeptide (MDP) 235
murine host
– population dynamics of bacteria 156
Mycoplasma pneumoniae 103, 254
Myeloid differentiation antigen 88 (MyD88) 48, 117, 129, 173, 236
myeloperoxidase (MPO) 22f.
– deficiency 27f.

n

NADPH oxidase 22ff.
Naip-5-dependent immune response
– cytosolic flagellin 130
NALP3 237
natural killer (NK) cell 226
necrotizing fasciitis (NF) 45
NEDD8 241
Neisseria
– *gonorrhoeae* 88, 302
– *meningitidis* 69ff., 85f., 255, 267, 301
NEMO 240
neonatal meningitis 79
– GBS strains 82
neuraminidase
– NanA 112
– NanB 112
– NanC 112
neurovascular unit 73
neutrophil
– antimicrobial mechanism 17
– function deficiency 26
– mature 17
– recruitment to sites of infection
neutrophil elastase (NE) 20ff.
neutrophil extracellular trap (NET) 17ff., 117
neutrophil gelatinase associated lipocalin (NGAL) 23
NOD-like receptor (NLR) family 236f.
non-druggable target 180
non-human primate 282
non-primate mammal 282
Nox2 ($gp91^{phox}$) 22ff.
Nramp1 152
Nramp1/Slc11a1 152
nuclear factor κB (NFκB) 33, 239
– pathway 236ff.
nuclear factor of activated T cell (NFAT) 35
nucleotide-binding oligomerization domain containing (NOD)
– NOD1 237
– *Nod1* gene 178
– NOD2 222ff.
– receptor 117

o

occluding 280
ompA 172
Opa protein 86
operational taxonomic unit (OTU) 196
organic cation/carnitine transporter (OCTN)-2 235
oxidative metabolism
– disorder 27

p

$p22^{phox}$ 22
$p40^{phox}$ 22
$p47^{phox}$ 22
p53 35
$p67^{phox}$ 22
p150,95 27
Paneth cell 222ff., 238
Panton-Valentine (PVL) leukocidin 57
partitioning defective 1 (PAR1)/microtubule affinity-regulating kinase (MARK) 35
pathogen
– infection and transmission 207
pathogen factor
– host 148
pathogen-associated molecular patterns (PAMPS) 166
pathogen–host relationship 167
pathogenicity island (PAI) 4ff., 81
– deconstruction and reconstruction 11
pathogenicity islet 115
pattern recognition molecule (PRM) 236
pattern recognition receptor (PRR) 48

PECAM-1 20
pelvic inflammatory disease (PID) 171
peroxisome proliferator-activated receptor gamma (PPARγ) 240
Peyer's patch (PP) 32, 140, 152, 216
phagocyte
– CD18-expressing 152
phagocytosis 17ff.
phagosomal–lysosomal fusion 125
pharyngitis 296
– *ex vivo* model 299
phenol-soluble modulin (PSM) 58
PhoP/Q regulon 140ff.
phosphoinositide kinase 22
phospholipase C (PLC) 22
phosporylcholine (ChoP) 110
phosphorylcholine esterase (Pce) 113f.
photoactivated localization microscopy (PALM) 270
phoX$^{-/-}$ mouse 153
phylotype 196ff.
phylum composition 200
PilA 84
PilB 84
PilC 84, 302
PilC1 88
PilC2 303
PilE 87, 302
pili
– type IV 86
PilQ 304
pilus 115, 280
pilus biogenesis 87
pilus-associated adhesin 84
PilX 88
plasminogen-binding group A streptococcal M-like protein (PAM) 297f.
Plasmodium
– *berghei* 283
– *falciparum* 283ff.
platelet activating factor (PAF) receptor 78, 110
pneumocinMN 111
pneumococcal colonization
– respiratory tract 110
pneumococcal disease
– epidemiology 106
pneumococcal genome 119
pneumococcal infection
– *in vivo* study 108
pneumococcal neuraminidase 112
pneumococcal surface antigen A (PsaA) 114
pneumococcal surface protein
– PspA 113
– PspC 110ff.
pneumococci 109
– influenza virus 111
pneumolysin 116
pneumonia
– sporadic and epidemic 119
poliomyelitis 286
polymeric immunoglobulin receptor (pIgR) 228, 300f.
polymorphonuclear leukocytes (PMN) 17
polymorphonuclear neutrophil (PMN) 46ff.
– avoidance of clearance 40
– evasion of migration 50
– interference of GAS and SA with PMN killing 54
– response 48
polysaccharide A (PSA) 208
polysaccharide capsule 112
Pontiac fever 119
PorA 304
positron emission tomography (PET) 255
proenzyme plasminogen (PLG) 297f.
protein kinase B (Akt) 235
protein kinase C (PKC) 22
proteinase-3 (PR-3) 23
Proteus 256
Proteus mirabilis 263
Pseudomona 263
PsicA 155
PsrP (SP1772) 116
PssaH 155
pyruvate oxidase (SpxB) 117

r

Rab GTPase 170
– family 24
RalF 123
reactive nitrogen species (RNS) 152
reactive oxygen species (ROS) 22ff., 56, 152f.
recombinase-based IVET (RIVET) 143
regenerating islet-derived-3γ (RegIIIγ) 202, 222f.
regenerating islet-derived-1α (RegIα) 33
Rel family 239
replication vacuole 126
respiratory tract
– pneumococcal colonization 110
reticulate body (RB) 168
retinoic acid (RA) 227ff.
RFX5 171
Rho 90
RNA interference (RNAi) 166ff.

– *Chlamydia*–host interaction 178
RNA-inducing silencing complex (RISC) 175
ROC-1 241
RrgA 111ff.
RrgB 114
RrgC 114

s
S100 protein family 57
salmochelin 8
Salmonella 256, 258ff.
– distribution 153
– *in vitro* cell model 141
– virulence gene 141ff.
– *enterica* 139, 283
– *typhi* 139ff.
– *typhimurium* 139ff., 281ff.
Salmonella pathogenicity island (SPI)
– SPI-1 140, 152ff.
– SPI-2 140ff., 153ff.
Salmonella-containing vacuole (SCV) 141, 262
Salmonella–mouse interaction 139ff.
secretory vesicle 23
Sel-1 126
selectin 19
seprocidin 23
SetA 123
SGD neutrophil 27
Shiga toxin 5
Shigella 256ff.
– *flexneri* 281ff.
SidM/DrrA 122
signature tagged mutagenesis (STM) 107, 144
SIGNR1 118
Simkania negevensis 167
sit operon 8
skin infection 45ff., 296f.
soft tissue infection 45ff.
solid tissue
– *ex vivo* infection 281
soluble *N*-ethylmaleimide-sensitive-fusion attachment protein receptor (SNARE) 24, 126f., 171
sortase
– SrtB 115
– SrtC 115
– SrtD 115
species specificity 279ff., 300
– genetic engineering 286
– molecular aspects 280
SpsA 110ff.
squalene synthetase (SQS) 57
Src homology 2-containing protein tyrosine phosphatase (SHP2) 35
staphylococcal complement inhibitor (SCIN) 56
Staphylococcus aureus (SA) 45ff., 110, 263
– avoidance of clearance by PMN 40
– restriction of cutaneous infection 46
Staphylococcus epidermidis 263
STAT3 33
stimulated emission depletion (STED) microscopy 270
stochastic optical reconstruction microscopy (STORM) 270
streptococcal C5a peptidase (ScpA) 50f.
streptococcal CXC chemokine protease (ScpC) 51
streptococcal infection 296
streptococcal inhibitor of complement (Sic) 299
streptococcal pyrogenic exotoxin B (SpeB) 299
Streptococcus agalactiae 69ff., 291
Streptococcus pneumoniae 69, 103ff., 254, 267, 300
Streptococcus pyogenes 281, 296ff.
– model of pharyngitis and skin infection 296
streptokinases (SK) 305f.
subarachnoidal spaces (SAS) 69
superintegron 5
surface capsular polysaccharide (CPS) 83
Syk 22

t
T cell 229
– balance in the gut 230
– cytotoxic 230
– effector 230
– natural T_{reg} (nT_{reg}) 231
– non-conventional 230
– regulatory (T_{reg}) 221ff.
T cell receptor (TCR) 230
T helper (Th) cell 229f.
– $CD4^+$T helper cell 229
– Th1 231
– Th1/Th2 balance 209
– Th2 231
– Th17 231f.
– Th17/T_{reg} balance 232
teichoic acid 111
tetracysteine-FlAsH labeling procedure 260f.
Tfp 88

thymic stromal lymphopoietin (TSLP) 221ff.
tissue type plasminogen activator (tPA) 297
TNF-α receptor 1 (TNFR1) 49
Toll-like receptor (TLR) 34, 48, 117, 173, 222ff., 263
– MAMP-TLR recognition 236
– TLR1 118
– TLR2 48f., 118, 236f.
– TLR3 78, 236f.
– TLR4 118, 233ff.
– TLR5 236f.
– TLR6 118
– TLR9 118, 232ff.
Tollip 239
Tom complex 178
total internal reflection fluorescence (TIRF) 270
toxic shock syndrome (TSS) 45
toxin gene 5
toxin-co-regulated pilus (TCP) 258
Toxoplasma gondii 268, 291
TP53 36
tracheal antimicrobial peptide (TAP) 240
transforming growth factor β (TGF-β) 221ff.
transgenesis 286
translocated actin-recruiting phosphoprotein (TARP) 172
transmigratory cup 90
transposon 4
Trefoil factor (TFF) family 220
tryptophan synthase (trpBA) 167
tumor necrosis factor (TNF) 20
tumor necrosis factor alpha (TNFα) 78, 225
twin arginine translocation (TAT) pathway 121
two-component system (TCS) 55
type three secretion (T3S) system (T3SS) 145, 259

u

Ubc12 241
ubiquitin degradation pathway (UPS) 241
ubiquitin ligase E3-$SCF^{\beta\text{-}TrCP}$ 241
UniFrac metric method 196
urinary tract infection (UTI) 9, 262ff.
urokinase type plasminogen activator (uPA) 297

v

vacuole protein sorting inhibitor protein (Vip) 122
– VipA 122ff.
– VipD 122ff.
– VipF 122ff.
VAMP 171
vancomycin-resistant *Enterococcus faecalis* (VRE) 52
VCAM-1 90
versatility
– bacteria 3
Vibrio cholerae 256
Vibrionaceae 256
villi 218
Virchow–Robin perivascular space 74ff.
Virchow–Robin space 71
virulence 125
– bacterial gene 146
virulence determinant
– ECNM strain 80
– GBS strain 83
virulence gene
– *Salmonella* 141ff.

w

wild-type isogenic tagged strain (WITS) 157
Wiskott–Aldrich syndrome related protein (WAVE2) 178

x

xenograft 284

y

ydiV/cdgR 153
yeast lethal factor A (YlfA) 123
Yersinia 256
– *pestis* 233
– *pseudotuberculosis* 289

z

ZmpB 115
ZmpC 115
ZmpD 115